SOME STUDIES ON MICROEMULSIONS AND THEIR UTILITY AS BIOCHEMICAL MODELS

A Thesis Submitted
In Partial Fulfilment of the Requirements
for the Degree of
DOCTOR OF PHILOSOPHY

By

C. KUMAR

to the

DEPARTMENT OF CHEMISTRY

INDIAN INSTITUTE OF TECHNOLOGY KANPUR

DECEMBER, 1979

STATEMENT

I hereby declare that the work embodied in this thesis entitled "Some Studies on Microemulsions and their Utility as Biochemical Models" has been carried out by me under the supervision of Professor D. Balasubramanian and Prof. P. Gupta-Bhaya.

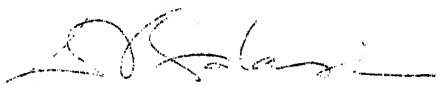
In keeping with scientific tradition, wherever work done by others has been utilised, due acknowledgement has been made.

C. Kumar

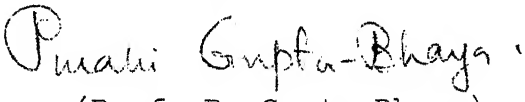
C. Kumar

CERTIFICATE I

Certified that the work presented in this thesis entitled, "SOME STUDIES ON MICROEMULSIONS AND THEIR UTILITY AS BIOCHEMICAL MODELS" by Mr. C. Kumar (Department of Chemistry, IIT-Kanpur), has been carried out under our supervision and has not been submitted elsewhere for a degree.



(Prof. D. Balasubramanian)
School of Chemistry,
University of Hyderabad,
Hyderabad-1



(Prof. P. Gupta-Bhaya)
Department of Chemistry,
IIT-Kanpur-208016

December 1979.

DEPARTMENT OF CHEMISTRY,
INDIAN INSTITUTE OF TECHNOLOGY KANPUR, INDIA

CERTIFICATE II

This is to certify that Mr. C. Kumar has satisfactorily completed all the courses required for the Ph.D. degree program. These courses include:

Chm 501	Advanced Organic Chemistry
Chm 521	Chemical Binding
Chm 523	Chemical Thermodynamics
Chm 541	Advanced Inorganic Chemistry
Chm 581	Introduction to Biochemistry
Chm 524	Modern Physical Methods
Chm 614	Organic Photochemistry
Chm 620	Frontiers in Biology
Chm 766	Experimental Biochemistry
Chm 800	General Seminar
Chm 801	Graduate Seminar
Chm 900	Graduate Research

Mr. C. Kumar was admitted to the candidacy for the Ph.D. degree in August 1974 after he successfully completed the written and oral examinations.

(Prof. S. Ranganathan)
Head,
Department of Chemistry,
I.I.T., Kanpur

D. N. Dhar

(Prof. D.N. Dhar)
Convener,
Departmental Post-Graduate
Committee, I.I.T., Kanpur-16

ACKNOWLEDGEMENTS

I humbly express here my sincere gratitude to

Professor D. Balasubramanian, for his kind interest, guidance, advice, encouragement and friendship during the entire course of my work;

Professor P. Gupta-Bhaya, for his assistance in many matters;

Professor P.T. Manoharan, Professor P. Balaram, Prof. P.M. Bhargava, Prof. D.O. Shah , Prof. J.P. Gupta and many other academicians for assistance, especially in utilising the experimental facilities at I.I.T., Madras, I.I.Sc., Bangalore, R.R.L., Hyderabad, and a few other laboratories;

Professor M.V. George, for caring;

My colleagues at the lab, here, in Hyderabad and in the many other laboratories that I have been a part of for a few days in the course of my vagabond research;

My colleagues at the Department of Chemistry, I.I.T., Kanpur, academic and other, for many happy moments;

My friends, for their kindness, indulgence and attempt to understand.

Authorities of I.I.T., Kanpur and the Science and Engineering Research Council for financial assistance.

C. Kumar

SYNOPSIS

Surfactant aggregates in nonpolar solvents have been studied in recent years with great interest, because of their special properties, as biomimetic catalysts, models for biological membranes, agents for enhanced oil recovery and encapsulating materials for enzymes etc. In nonpolar media, such aggregation leads to reverse micelles with a polar interior and nonpolar outer surface; and, as a result, such aggregates can solubilise water in their interiors as small water pools. Traditionally such systems are classified into two types: (a) reverse micellar systems consisting of a pure surfactant in an organic solvent as the continuous phase and small amounts of water as the dispersed phase, and (b) microemulsions consisting of a primary surfactant, a secondary surfactant such as an alcohol, organic solvent and water. While a few reverse micellar systems and anionic microemulsions have been extensively characterised by past workers, studies on nonionic microemulsions have been few. The first part of this thesis concerns itself with the characterisation of a nonionic microemulsion system utilising the nonionic surfactant, Triton X-100.

After a review of the relevant literature in the field of microemulsions in the Introduction, we present data in Chapter 2, on the hydrodynamic and structural characterisation of the microemulsion system Triton X-100 (p-octylphenyl ether of nonaethyleneglycol)-alcohol-cyclohexane-water. By a study

of the water uptake values of solutions of Triton X-100 : alcohol mixtures of different ratios in cyclohexane, it is shown that at the optimum Triton X-100 : alcohol ratio of 4:1 w/w, about 10%v/v of water is solubilised by a 20%w/v solution of the surfactant mixture in cyclohexane to yield clear microemulsions. Further addition of water, upto about a total water concentration of 17%v/v, caused the system to change into a viscous, thixotropic and optically anisotropic liquid crystalline phase while beyond 17%v/v water, phase separation occurs. The microemulsion and liquid crystalline phases and the transitions between the two have been characterised by electrical conductivity, optical birefringence, viscosity, electron microscopy and light scattering methods. The microemulsions are shown to consist of spherical water pools ($<$300 Å radius) surrounded by surfactant films, while the liquid crystalline phase consists of large (about 2000 Å radius of gyration) lamellar structures. These studies have been carried out for different alcohols, namely pentanol, hexanol and octanol. The fraction of alcohol present at the interface and the Free energies of transfer of the alcohols from the bulk to the interface also have been estimated for these different alcohols.

In Chapter 3, we describe in detail spectroscopic studies on this system in the microemulsion and lamellar phase and analyse the state of water in the entrapped pools. Near infrared spectroscopy shows that most of the water resides in the water pools and only a negligible fraction in the continuous

phase. The electronic absorption band of NO_3^- , used as a polarity probe, reveals that the polarity of water pools increases from an initially low value to that of bulk water with increasing water concentration. This conclusion is supported by the fluorescence emission wavelength, quantum yield and depolarisation values of 8-anilinonaphthalene sulfonic acid, a fluorescence probe used in the system. $CoCl_2$, used as a probe to study the state of water in the water pools, exists as the blue tetrahedral complex at low water concentrations and changes to the octahedrally hydrated species only at higher water concentrations, showing that water in the pools is mostly bound to the surfactant at low water concentrations. Quantitative estimates of bound and bulk water as well as the mobility of the water and surfactant residues of the system are presented through a study of the ^{1}H-NMR relaxation times T_1 and T_2 of the ethylene oxide and OH protons of the system. The fluidity of the surfactant layers at the alkyl aryl end of the ethylene oxide chain has been studied by monitoring the rotational correlation time of a spin probe, 2,2,6,6-tetramethylpiperid-4-one N-oxide-2,4-dinitrophenylhydrazone dissolved in the system at different water concentrations. The implications of these results to the formation and the structure of nonionic microemulsions and liquid crystalline structures are discussed.

The rest of this thesis concerns itself with the utilisation of microemulsions as models for some interesting biological systems. Utilisation of microemulsions for this purpose leads

to many advantages because our nonionic systems are optically transparent, easily made, avoid charge effects and allow ordering of substances of interest at the molecular level.

In Chapter 4, we present a study of the conformation of bovine serum albumin and of α-chymotrypsin included into the water pools of different microemulsions. These proteins are found by circular dichroism studies to preserve their native structure in microemulsions made from sodium dodecyl sulfate and from decaoxyethylene cetyl ether. The activity of the encapsulated enzyme α-chymotrypsin in these systems as well as the Triton X-100 system are presented at different H_2O concentrations and support the conclusions drawn from circular dichroism studies. In contrast, these proteins are found to be denatured when included into microemulsions of sodium laurate, cetyl trimethylammonium bromide or sodium dioctyl sulfosuccinate in nonpolar solvents. The implications of microemulsion encapsulated enzymes for synthesis and as cellular models are discussed.

In the last chapter of the thesis, we present results on the use of a liquid crystalline lamellar system as a model photoregulatory membrane. The model system consists of a photoisomerisable compound, azobenzene, incorporated into the lamellar liquid crystalline phase of the system potassium oleate, hexanol, hexadecane and water. When the imbedded azobenzene is photoisomerised, it causes changes in the molecular organisation of the lamellae, as shown by bulk conductivity measurements.

It is further demonstrated that the activity of an enzyme, α-chymotrypsin, included into this system, shows significant changes when the imbedded azobenzene is photo-isomerised, providing a model for the photoregulation of enzyme activity in biomembranes.

CONTENTS

-*-

LIST OF FIGURES

List of Figures (contd.)

List of Figures (contd.) Page

LIST OF TABLES

CHAPTER I

I N T R O D U C T I O N

The study of biological membranes has achieved a central position in biochemical enquiries into the structure of living organisms in the past two decades. Other than the task of simply delineating the physical boundaries of cells and cellular organelles, it has been established that the membranes fulfill many other vital functions. Membranes are the loci at which transport of materials into and out of the cells is controlled. Many important systems, like the photosynthetic and immunological systems are intimately linked with the membranes that they are built into. Membrane-bound centres are also the site of protein synthesis. Excitable membranes are also the medium of nerve conduction.

Considering the complex nature and number of functions that the membranes are called on to perform, it is no wonder that the structure of the membranes is also a complex problem to unravel. The two structural elements of membranes are proteins and lipids. The diverse nature of the proteins and lipids found in natural membranes makes the study of their various interactions a difficult task. Nevertheless, insight into this problem has been developed and the contributions made by the numerous workers in this field have led to coherent models of the organisation of natural membranes - the protein mosaic model (1,2).

One important structural feature of these models is the presence in the membrane of large lipid regions which have almost no protein as part of them (3). The nature of lipid

organisation in these bilayer areas are amenable to investigation in model systems that are simpler than the natural membranes. Two such model systems are the liposomes (4) and the black lipid membranes (5) which have succeeded the surface monolayers (6) as more realistic model membranes.

Liposomes are spherical bilayer vesicles that are formed spontaneously when dry lipids are dispersed in aqueous buffers. They may consist of multibilayers when dispersion is through agitation or be single walled as when the lipid is dispersed through ultrasonication. Spin label, NMR and fluorescence studies on these systems have contributed enormously to our knowledge of lipid-lipid and lipid-protein interactions. From a study of liposomes and natural membranes is also derived the understanding of the temperature dependent gel to liquid crystalline phase transitions of lipid assemblies (7).

Black lipid membranes (BLM) are lipid bilayer assemblies that are formed when a lipid solution in an organic solvent is applied onto a small (1 mm radius) hole in a polymer support immersed in an aqueous buffer. These systems are stable normally for a few hours. While not amenable to spectroscopic studies easily, BLMs are excellent models for studying the electrical characteristics of lipid membranes.

Consideration of the amphiphilic nature of the lipid molecules have led many workers to probe into the nature of intermolecular interactions within aggregates of synthetic surfactants in different solvents. The interest in surfactant assemblies

arises because the forces that control the formation of surfactant aggregates are similar to those responsible for the formation of lipid assemblies.

Of course, there exists a large body of literature on surfactant aggregates due to the intrinsic academic and industrial interest in micelles per se. However, the search for model membrane systems have led to the development of another point of view, an additional source of interest in surfactant aggregates.

The similarities between surfactant aggregates and biological structures are as follows:

(1) The hydrophobic forces that lead to the association of the hydrocarbon tails of lipids, and the dipole-dipole and/or ion-dipole interactions amongst the head groups and between them and the solvent are the same as those that control micelle formation.

(2) The micelle-solvent interface is in many ways similar to the lipid membrane-solvent interface.

(3) Surfactant assemblies show changes in the rigidity of their organisation upon chemical or thermal perturbation similar to lipid membranes.

(4) Surfactant assemblies are capable of catalysing many reactions and promoting interactions in a way similar to membrane interfaces and enzyme-solvent interfaces.

Due to the above reasons, attempts have been made in the past to develop and study model membrane systems based on surfactant aggregates, and the work described in this thesis is a contribution in this direction.

Surfactant aggregates can be broadly classified into three categories, micelles, reverse micelles and microemulsions. These divisions are basically traditional and many other aggregate forms like the liquid crystalline phases may either be classified distinctly or be considered in continuation with one of the three above types.

Micelles, the multimolecular aggregates of surfactants in water (and occasionally other polar media), are the basis of such a large number of studies that to review them here would be redundant. In short, micelles are formed by the aggregation of surfactants through hydrophobic interaction. Micellar aggregation occurs above a particular concentration of surfactant in solution, known as the critical micelle concentration (cmc). However, it has been suggested that an indefinite self-association model of stepwise aggregation may be more plausible (8). The cmc as well as the aggregation number (AN) are well known to be sensitive to additives like urea, alcohol etc. (9, 10). Also at high concentrations of surfactant, at more than 20 times the cmc, the micelles enlarge and aggregate, ultimately to produce liquid crystalline phases (11). Micelles are also capable of solubilising small amounts of nonpolar compounds.

The exact microstructural features of these micelles has been a subject on which clarity has been lacking. Initially, McBain had proposed box like double leaflet structures (12) which was superseded by a spherical micelle with a hydrocarbon interior and a smooth, hydrophilic surface (13). The spherical micelle or an ellipsoidal variant has been the long standing, accepted model.

Recently, Menger (14) has reviewed data relevant to the structure of ionic micelles and has shown that a pincushion like structure may explain the known facts about micelles better. In the model discussed by him, a small (10-15% of micelle volume) nonpolar core is formed by the coming together of the nonpolar ends of the surfactants, with the rest of the surfactant molecule projecting out of this core into the surrounding, essentially aqueous solvent. The distinctive feature of this model is the existence of deep grooves in the micelle which normally are filled by water. These grooves may act as the centres where nonpolar organic compounds are solubilised, rather than by simple dissolution in the small nonpolar cores of the micelle.

Another interesting aspect of the micellar aggregates is their catalytic properties. Both due to favourable partitioning and orientation of the reactants, as well as the presence of large interface areas, micelles act as efficient catalysts for many reactions (15,16). It has been suggested that the saturation kinetics shown by micelles may be similar to the saturation kinetics of enzymes. Also, there are structural similarities

between a substrate at a micellar interface and at the enzyme active site. Both of these situations hold the substrate in an aqueous environment on one side while the other is in contact with an organic environment. Further, in charged micelles, the charges on the micelle surface may interact with the substrate in an enzyme-like manner (15,16).

The interactions between surfactant molecules in micelle formation, especially the hydrophobic interaction, has been likened to the forces responsible for the formation of lipid membranes (17). Also, the interaction of surfactants with proteins has been used as a model for lipid-protein interactions in the natural membrane (18,19).

Complementary to the surfactant micelles in aqueous media, surfactants are known to aggregate extensively in non-polar media also. Presumably, in nonpolar media, reverse micelles are formed on aggregation of the amphiphile through their polar head groups, with their hydrophobic tails projecting out into the bulk solvent. Such reverse micelles are able to solubilise significant quantities of polar solvents, specifically water. Studies relevant to the structure of the reverse micelles and the nature of the water solubilised in them have been reviewed (20,21).

The most thoroughly studied reverse micelle systems are Aerosol OT (sodium bis(2-ethylhexyl)sulfosuccinate), dodecylammonium propionate and partly, Igepol CO-530 (polyoxyethylene-(6)-nonylphenyl ether) systems.

The aggregation of Aerosol OT and other surfactants in nonpolar solvents has been studied by various techniques (22, 23,24). It has normally been found that many properties of such solutions show a break at a particular concentration of surfactant that can be termed the operational critical micelle concentration. Though in the dry system there may be no surfactant association, the addition of polar substances like water may produce association or change the aggregation number (20,25). It has also been suggested that presence of water may be essential for the production of reverse micelles (26). However, it has been observed that water prevents association of polyoxyethylene type surfactants in nonpolar solvents (27). This would seem to be an unusual exception considering that in many cases water is necessary for the formation of reverse micelles (26).

In general, the size of the aggregates produced in nonpolar solvents is rather small, the aggregation number being 2 to 40 (23,27). The small aggregation number would seem to be a direct consequence of the spherical structure and the exact value of the aggregation number depends on the size of the hydrophobic part of the surfactant (28-30). However, the whole approach to this problem of aggregation numbers has been questioned (31,27) and the experimental data interpreted in terms of indefinite self-association of the surfactant, rather than by assuming the formation of micelles of a certain size and specific aggregation number.

Other than the studies on simple solutions of surfactants in dry nonpolar solvents, solubilisation of water into nonpolar

solvents by reverse micelle forming surfactants has been investigated extensively (20). Aerosol OT, for example is capable of solubilising upto 50 moles of H_2O/mole surfactant in its reverse micelle water pools (32). Of this solubilised water an initial portion goes to hydrate the cation, about 6 water molecules being thus immobilised by each surfactant molecule (33). A part of the water solubilised into the Aerosol OT reverse micelles, perhaps due to interactions with the polar head groups of the surfactant, loses its dissolving capacity for inorganic salts (34). The state of water solubilised in Aerosol OT reverse micelles has been investigated extensively by spectroscopic methods and the results are as follows:

(1) Fluorescence studies utilising ANSA (8-anilinonaphthalene sulfonic acid) as a fluorescence probe showed the polarity of water solubilised in Aerosol OT to be much lower than that of bulk water. The polarity slowly increased with water concentration to values more near normal (35).

(2) NMR studies showed water to be present in a rigid, non-hydrogen bonded environment at low concentrations in Aerosol-OT/heptane solutions. Rigidity decreased and H-bonding increased as the hydration of the Na^+ cations in the reverse micelles was completed. Further additions of water shifted the properties of water in these reverse micelles to near those of bulk water (36).

(3) Spin label studies show that certain molecules that may dissolve in the core of the water pool in Aerosol OT reverse

micelles at high water concentrations may be forced out of the aqueous phase, into the interface, at lower water concentration (37).

(4) A study of the pK_a of p-nitrophenol dissolved in the water pools in Aerosol OT in heptane showed the pK_a of p-nitrophenol to be shifted to above 11 from its normal pK_a of about 7.4 in water. The shift in pK_a could be assigned specifically to adsorption of p-nitrophenol at the interface (38).

(5) The Kosower polarity index, Z value of water solubilised in Aerosol OT reverse micelles lies in between water and methanol at low water concentrations, but the Z value changes at high water concentration to values near those of bulk water (39).

Similar results have been obtained for acid ammonium propionates (40,41) and some polyoxyethylene surfactants (42-44). Further, it has been pointed out that though the decrease in polarity and fluidity of water is seen in most reverse micelle systems, the pH of solubilised water may or may not be different from bulk water (20). This is one of the properties of solubilised water that differs from system to system. Polyoxyethylene-nonylphenyl ether solublised water was found to be highly acidic while dodecylammonium propionate solubilised water was found to be similar to bulk water by measuring the pK_a of water soluble dyes dissolved in water pools (20).

Lecithin forms reverse micelles in ether that solubilise significant quantities of water. Part of the water is bound to

lecithin headgroups and is unavailable to hydrate Co^{2+} ions included in the water pool (45). Other lamellar aggregates of lecithin in nonpolar solvents in presence of water are also known (46).

Other than the simple reverse micelles, most surfactants form much larger aggregates at high concentrations in nonpolar solvents, in presence of some water. Often these phases are liquid crystalline and some studies on such phases have been reviewed (23,24).

The recent interest in reverse micelles has been largely due to their catalytic properties (15,16). Reverse micelles containing solubilised water are known to catalyse many reactions very efficiently due to proper partitioning and orientation of reactants. The kinetics of these systems resembles enzyme catalysed reactions in some respects and similar mechanisms have been proposed (20). Reverse micelle enclosed water has also been likened to water found in membranous packets in biosystems like mitochondria, especially in the presence of the bound water species in these systems (36,47). Enzymes included into reverse micelle water pools have been used as models for cellular enzymes (48,49). Other than these points, the reverse micellar systems are of considerable industrial importance as well (50).

The conditions for increased solubilisation of water into nonpolar solvents and of nonpolar molecules into water by surfactants have been investigated (51-57). In these studies it

was shown that for every surfactant in a given water - non-polar solvent system, there exists a particular optimum temperature at which the solubilisation capacity of the surfactant is very high. Above and below this temperature, the solubilisation capacity is much lower. This temperature is directly related to the Phase Inversion Temperature of the surfactant in the case of nonionic surfactants. By mixing two surfactants of dissimilar hydrophile-lipophile balance (HLB) values, we can arrive at optimum combinations that possess good solubilisation efficiency at any given temperature. Larger size of surfactants leads to better solubilisation and mixtures of nonionic and ionic surfactants show good temperature stability of their solubilisation capacities. The efficiency of solubilisation of water into hydrocarbon is dependent upon counterions and dissolved salts (54).

An intuitive grasp of the factors involved in the solubilisation efficiencies led Schulman to the formulation of the first high solubilisation efficiency system, which later came to be called microemulsion. Since the main body of this thesis concerns itself with the properties of nonionic microemulsion systems, we now present a brief review of relevant past work in this field.

Microemulsions are systems consisting of water, a nonpolar solvent, a surfactant and cosurfactant. In general an alcohol is used as the cosurfactant. One of the two liquids forms the continuous phase and the other is contained in small (circa 100 Å

radius) spherical droplets, surrounded by a mixed film of surfactant and cosurfactant. Depending on whether oil or water forms the continuous phase, the system is called a water-in-oil or oil-in-water microemulsion , respectively. Many microemulsions of one type can be converted to the other, i.e., water-in-oil to oil-in-water, by adding increasing quantities of one of the phases. Normally, this inversion occurs through an intermediate viscoelastic gel stage. In microemulsions, the cosurfactant alcohol is considered to be distributed between the bulk phase and the interfacial surfactant film. Microemulsions appear to be clear solutions because due to the small size (compared to the wavelength of visible light) of the dispersed phase droplets, the system is only weakly light scattering. The exact phase boundaries of microemulsion systems are sensitive to counterions as well as temperature. Also, for any given surfactant, different cosurfactants seem to produce different results in terms of their solubilisation efficiency, size of the microemulsions produced and phase boundaries. The work done so far on microemulsions have been recently reviewed by Shah (59) and Prince (60).

The investigations into the nature and formation of microemulsions started with the work of Schulman and his coworkers (58). Hoar and Schulman (58) studied water-in-oil microemulsions and formulated the following conditions for their formation with soaps as primary surfactants: (1) A high soap to water ratio, (2) Presence of a nonionic cosurfactant, alcohol, acid

or amine, of a size smaller than that of the soap at molar concentrations close to that of the soap. They suggested that spontaneous emulsification of added water into small droplets occurred in these systems because of the near zero interfacial tension between water and oil due to the effect of the surfactant mixture. Schulman and McRoberts (61) found that hexanol and longer alcohols were effective as cosurfactants in water-oil systems in producing water-in-oil microemulsions with soaps as primary surfactants. Alcohols of lower chainlength produced conducting solutions of indefinite structure.

Based on X-ray and light scattering measurements, Schulman and coworkers (62,63) showed the oil-in-water and water-in-oil microemulsions to consist of small (100-500 Å radius) spherical droplets of the dispersed phase, surrounded by a 20 Å thick surfactant film. The radius of the dispersed phase droplets decreased with increasing surfactant concentration. Similarly, the size of the dispersed phase droplets was sensitive to both the surfactant-cosurfactant ratio and the total concentration of the dispersed phase. Moreover, the microemulsions seemed to consist of closely packed dispersed phase spheres in a medium of the continuous phase.

In order to effect comparison, Schulman (65) also studied the structures formed by high concentrations of nonionic, polyethylene oxide type surfactants in oil/water. They showed the formation of lamellar, cylindrical and spherical structures in these surfactants, depending on the nature of both the polar

and nonpolar part of the surfactant.

In 1955, Bowcott and Schulman published a landmark paper (66) which discussed many aspects of microemulsion formation. They found that K-oleate/water/benzene with butanol or pentanol, at low water concentrations produced highly conducting 'anomalous' systems, while hexanol produced nonconducting water in oil microemulsions. The hexanol was found to be distributed between the interface and bulk organic phase, while the soap was assumed to be present in the interface entirely. Estimates of hexanol at the interface showed the alcohol to soap ratio at the interface to vary between 2:1 to about 3.2:1. Substitution of hexanol by longer chainlength alcohols increased the size of the microemulsions produced.

Bowcott and Schulman suggested that the interfacial surfactant film can be considered to be a separate phase (interphase). This film has different properties at its two interfaces, one with oil and the other with water. The two interfaces have different interfacial tensions and their resultant determines the curvature, size and phase continuity of the microemulsions. The function of the alcohol is to penetrate the interfacial soap film and increase the disorder present in the film. This has the effect of turning the soap film into a two dimensional liquid, with different interfacial tension values on its hydrophilic and hydrophobic sides. If the interfacial tension between the surfactant film and oil ($\gamma_{m/o}$) is greater than the interfacial tension between the surfactant film and water ($\gamma_{m/w}$)

then an oil-in-water microemulsion is produced. If $\gamma_{m/o} < \gamma_{m/w}$, then a water-in-oil microemulsion is produced.

Following their electron microscopic observations on microemulsions (67,68), Schulman, Stoecknius and Prince suggested that the low (near zero) interfacial tensions required for the formation of microemulsions could be achieved by increasing disorder in the interfacial soap film through (a) penetration of the interfacial mixed surfactant film by hydrocarbon molecules, (b) use of large counterions to make the ordered packing of soap molecules in a two dimensional film difficult and (c) easy penetration of the soap film formed according to (b) by molecules from the oil phase. Support for these notions came from the previously available studies on penetration of surfactant monolayers by oil molecules at the oil-water interface (69,70). It was also pointed out (66) that the phase continuity of the microemulsions could be affected by surface charges on the surfactant film, a charged film favouring the formation of an oil-in-water microemulsion.

Schulman considered the condition for stability of microemulsions to be zero interfacial tension between oil and water at equilibrium, with spontaneous microemulsification being due to transient negative interfacial tension. This concept of transient negative interfacial tension has been extended by analogy to other systems by Davies and Haydon (71).

Molecular interactions at the interfacial film and the interfacial tension in microemulsion systems have been discussed

by a number of authors (72-78). The thermodynamics of such systems has been reviewed recently (79,80).

The early studies of Schulman essentially concentrate on the physical properties and stability of the microemulsion systems. Spectroscopic work on these systems has been carried out extensively by Shah and coworkers (59,81-85). Conductivity, NMR and viscosity studies were used to establish that water-in oil microemulsions are converted on addition of water to oil-in-water microemulsions through intermediate stages of water cylinders and water lamellae in the system water, hexadecane, potassium oleate and hexanol. They also established that the lamellae in the liquid crystalline phase could be ordered with respect to each other on standing. Shah has also suggested (85) that the lamellar phase of the above system may be utilised as a model membrane since it responds to calcium ions and anaesthetics in a way similar to lipid membranes. The importance of the above work lies in the experimental demonstration of the sequential appearance of different kinds of association structures in the surfactant system. The sequence of structures previously predicted by Winsor theoretically are shown in Fig. 1.1 (86). Further, Shah and coworkers presented NMR evidence, alongwith conductivity data, to show that when pentanol is used as cosurfactant instead of hexanol, the potassium oleate, water, oil system produces molecularly dispersed (or cosolubilised) water-in-oil systems, rather than water-in-oil microemulsions. However, it has later been shown that even in pentanol

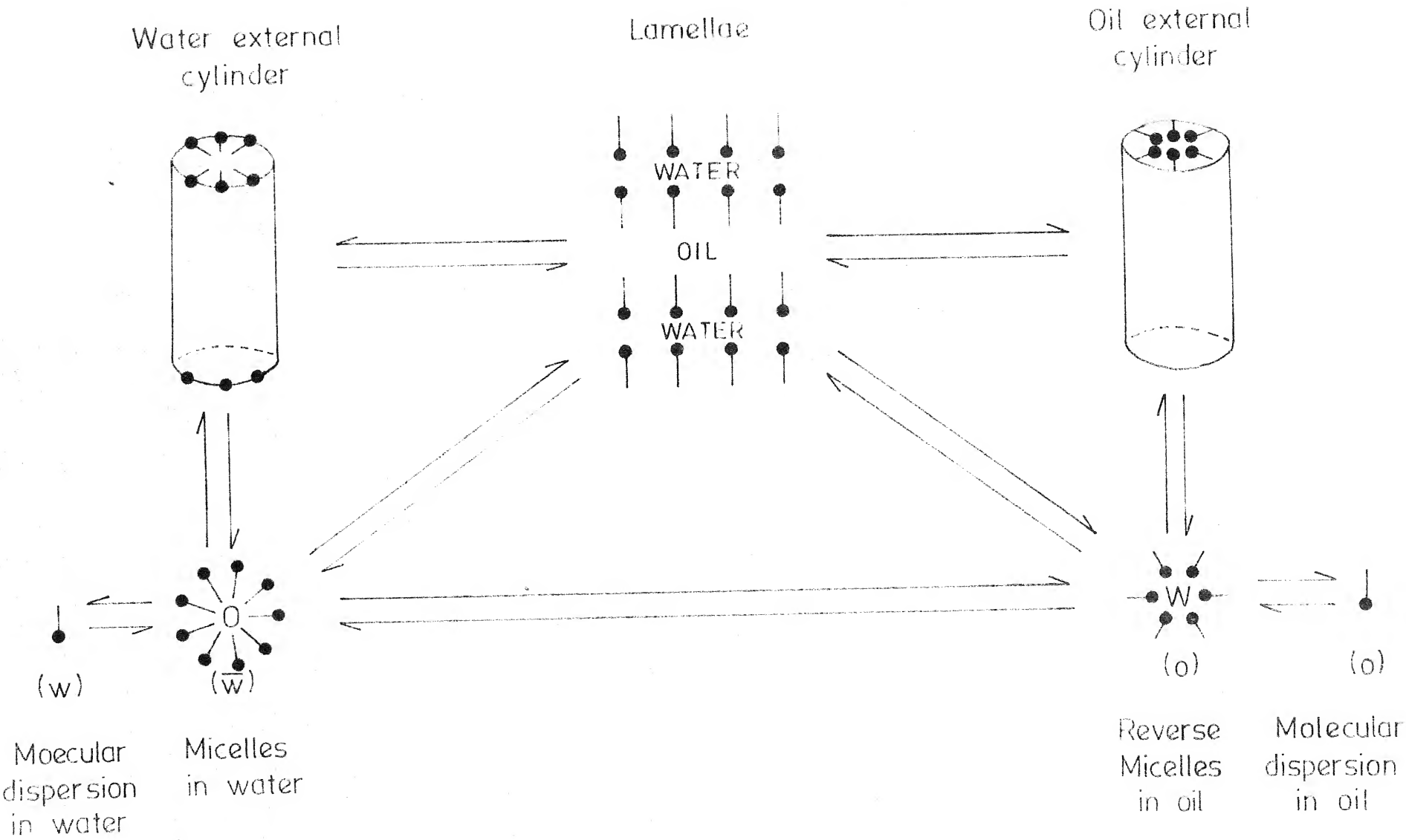


Fig. 1.1 Equilibria in the surfactant / water / oil system.

cosurfactant systems, microemulsions are produced (87).

Rosano and coworkers, investigating the conditions of microemulsion formation have shown that alkyl sulfates form microemulsions irrespective of the counterion, while soaps are counterion sensitive(88). Gerbacia and Rosano (89) studied the distribution of the alcohol between the interface and bulk phase. They also suggested that the formation of microemulsions was sensitive to the order of addition of components, a statement that has been later questioned (90). Rosano and coworkers have interpreted the results of their experiments to indicate that micromulsions are kinetically, rather than thermodynamically, stable (91,92). Kai-Li-Ko has examined the soap-alcohol-oil-water system by light scattering (93).

Combining together the results of studies like those carried out by Shah, Prince has proposed a phase diagram of surfactant-oil-water systems which is presented as Fig. 1.2 (94). The interesting aspect of this hypothetical phase diagram is the way it unifies micellar solutions, o/w microemulsion, lamellae, cylinders, w/o microemulsions and reverse micellar solutions into a rigid sequence.

From a study of ternary and quarternary phase diagrams, an approach pioneered in this field by Ekwall (95,96), Friberg has questioned the validity of the term microemulsions at all (97-99). Friberg points out that the microemulsions are a natural continuation of the reverse micelle region in a water-surfactant-cosurfactant phase diagram when a fourth component,

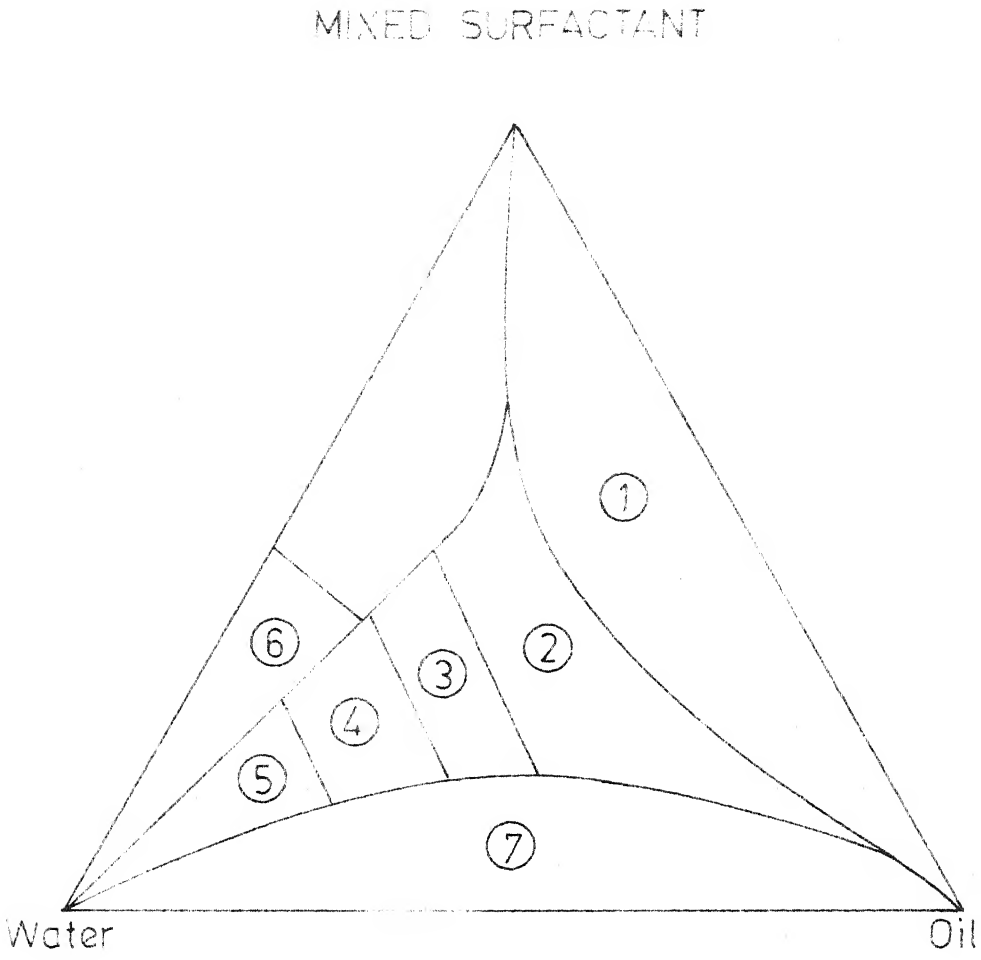


Fig. 1.2 Phase diagram according to Prince

1. Reverse Micelle
2. W/O microemulsions
3. Cylinders
4. Lamellae
5. O/W microemulsions
6. Micelles
7. Emulsions

oil, is added to it. From considerations of 4-dimensional phase diagrams for the normal microemulsion systems, he formulates a phase diagram similar to Fig. 1.3, very different from the Prince phase diagram, Fig. 1.2. As a consequence of this, the liquid crystalline phases found during inversion of microemulsions at intermediate water to oil ratios will fall in the multiphase region of this phase diagram. The apparent phase stability of these liquid crystalline phases may be only kinetic. In this context the use of the special term 'microemulsions' has been questioned and 'swollen micellar solutions' considered more appropriate.

Bansal and Shah (59) have suggested that 'microemulsions' may be a class of aggregates with a significant enough distinction even though they may be simply swollen micellar solutions because (a) while all microemulsions are swollen micellar solutions, not all reverse micelles can be swollen with a solubilisate to the size level of microemulsions, (b) the higher solubilisation capacities and larger sizes of aggregates found in microemulsions distinguish them quite significantly from simple swollen micelles and reverse micelles.

Nevertheless the basic controversy can be settled only when significant thermodynamic or supramolecular organisational differences can be established between microemulsions and swollen micelles, or the absence of such differences is established. Until then, as is the scientific custom, convention is likely to prevail and the traditional term 'microemulsions

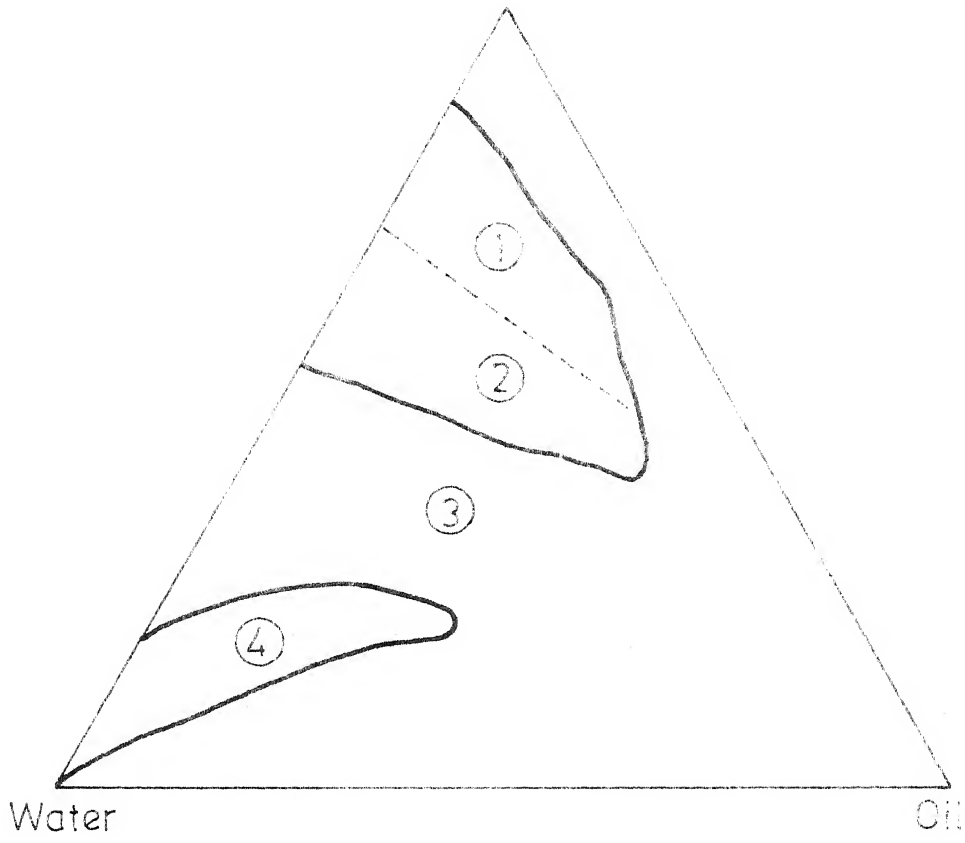


Fig. 1.3 Phase diagram according to Friberg

(1) Solubilised System
(2) Reverse Micelle
(3) Multi phase region
(4) O/W Micellar solution

retained for surfactant-cosurfactant-oil-water systems of the size and shape already discussed.

Our interest in the microemulsion systems is due to the idea that these systems can be developed into interesting models for biochemical systems.

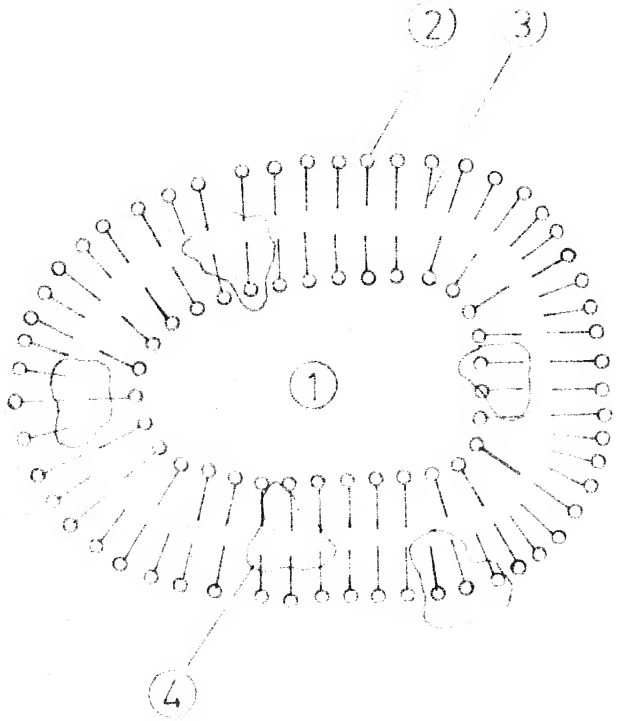
In cells and many subcellular organelles, membranes that define the boundaries of the system extend their influence on the entire system. In many organs, membranes are folded such that they possess a very large interfacial area. Proteins may interact directly with the lipid or protein components of the membranes. Moreover, and more importantly, the membrane components can interact with the water in the cell in specific ways through their headgroups, affecting water structure and consequently the structure of macromolecules dissolved in cellular water. Further, the membrane acts as a barrier to transport of matter into and out of the cell system. The bilayer lipid membrane surrounding the aqueous central compartment of the cells is supremely well organised to fulfill its biochemical functions.

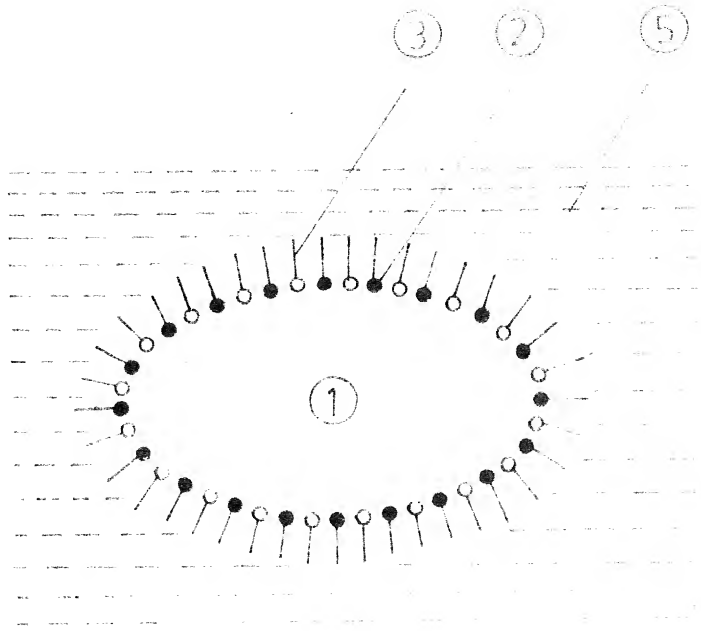
From the centre of water pool, a large number of similarities can be seen between water-in-oil microemulsions and the cell as a whole. The bilayer lipid membrane in the cells is represented by the mixed surfactant interfacial film of the microemulsions. The central water pool of the w/o microemulsions is similar to the water in the cell. The lipid membrane presents a surface sheath of polar head groups, backed

by a strongly hydrophobic nonpolar region, when viewed from the inside of the cell. A similar arrangement of polar groups, backed by a hydrophobic region, is achieved in w/o microemulsions as well. The formal similarity of the core of water-in-oil microemulsions to cellular environments is depicted in Fig. 1.4.

While reverse micelles with some solubilised water in them may also be similar to w/o microemulsions in these respects, the small size ($<$ 100 Å diameter) of the reverse micelles is a point against their favour. The much larger size of the water pools in microemulsions formally make them closer to the natural dimensions of cells and hence, they may be better models of cellular environments.

A few studies have been reported in literature in which microemulsions have been utilised for similar purposes. Shah (85) as mentioned earlier, showed the effect of Ca^{2+} and anaesthetics on soap-hexanol systems to be similar to their effects on biomembranes. A microemulsion system has been utilised to study the mechanism of interaction of Cu^{2+} ions with ligands at a polar-nonpolar interface as it occurs in different enzymes (100). A few biochemically important reactions have been studied at microemulsion interfaces by Mackay and coworkers (101 - 105). The reactions studied were: (1) incorporation of divalent metal ions by tetraphenylporphine in terms of kinetics and intermediates, (2) photodegradation of chlorophyll, (3) chlorophyll sensitized photoreduction of dyes by ascorbate and





Schematics of

A cell

w/o microemulsion

(1) Water (2) hydrophilic head group (3) hydrophobic tails (4) Protein (5) oil

Fig. 1.4

(4) acid-base equilibrium, dye absorption and reactivity. Most of these studies were carried out on oil-in-water microemulsions in the system sodium cetyl sulfate-alcohol-hydrocarbon-water.

We have concentrated in the initial part of this thesis on water-in-oil microemulsions of the nonionic system Triton X-100 - alcohol-cyclohexane-water. The nonionic system was chosen because

(1) with a nonionic microemulsion charge interactions being absent, the weaker dipole-dipole interactions between water and polar groups in water-in-oil microemulsions are studied better. Similarly, when biopolymers like enzymes are included into a nonionic microemulsion, absence of charge interactions may make interpretations easier and less ambiguous.

(2) In the absence of ionisable groups lining the water pool, the definition and control of pH of the water pools is more concrete. This is an important property when biochemical modelling is envisaged as a purpose of the study.

(3) Triton X-100,* ~~polyoxyethylene~~ is a widely used biochemical reagent whose aqueous micelles are well studied (106,107). Hence our microemulsion studies will be complementary to the aqueous micelle and reverse micelle studies on similar systems.

(4) There is a lot of intrinsic interest in the complete characterisation of a nonionic microemulsion system since past

* p-tert-~~octyl~~ octyl ether of polyethyleneglycol 9-10.

studies have been essentially concerned with ionic microemulsion systems. Further, while reverse micelles have been extensively studied and spectroscopically characterised, similar studies in depth of the spectral properties of microemulsion systems have not been carried out. Most work on microemulsions, as reviewed earlier, has been carried out on properties like conductance, viscosity, light scattering, phase diagrams, etc. In the light of the 'swollen micellar solution' versus 'microemulsion' controversy, we believe that spectral characterisation of microemulsion systems utilising techniques developed for the study of reverse micelles will be of value.

(5) In the case of w/o microemulsions, by adjusting the HLB value of the surfactant by altering the surfactant-cosurfactant ratio, the amount of water solubilised can be increased to high values easily at any given temperature. Such a feature is not characteristic of reverse micelles. Normally all reverse micelles, especially nonionic reverse micelles, have low aggregation numbers and are of a small size which makes them unsuitable for our present purpose.

With the above considerations in mind, we have, in the first part of the thesis, characterised the microemulsion and liquid crystalline phases of the Triton-X-100 - alcohol-cyclohexane-water system. Chapter II deals with studies directed towards the establishment of phase boundaries and solution properties of the surfactant aggregates. Chapter III discusses

the results of spectroscopic studies on these systems. Many spectroscopic techniques are applied to the microemulsion systems for the first time to the best of our knowledge and, we believe, with good results.

Chapter IV and V discuss the utilisation of microemulsions for simple biochemical modelling purposes. Chapter IV deals with the activity and conformation of proteins included into water-in-oil microemulsions and presents such systems as models for enzymes included in cells. Chapter V deals with the utilisation of a microemulsion forming system as a model membrane with photoresponsive properties. We also discuss a novel model for the photocontrol of biological systems in general in Chapter V. The past results relevent to these studies are reviewed in the beginning of Chapter IV and V.

REFERENCES

1. S.J. Singer, p. 145, 'Structure and Function of Biological Membranes,' (Ed. L.I. Rothfield), Academic Press, New York, 1971.
2. 'Functions of Biological Membranes,' M. Davies, Chapman and Hall, London, 1973.
3. A.D. Bangham, Ann. Rev. Biochemistry, 41, 753 (1972).
4. A.D. Bangham, M.W. Hill and N.G.A. Miller, p.1, 'Methods in Membrane Biology,' Vol.1 (Ed. E.D. Korn), Plenum Press, New York, 1974.
5. 'Bilayer Lipid Membrane,' H.T. Tien, Marcel Dekker, New-York, 1974.
6. N.L. Gershfeld, p. 68, 'Methods in Membrane Biology,' Vol.1 (Ed. E.D. Korn), Plenum Press, New York, 1974.
7. R.A. Nystrom, p. 44, 'Membrane Physiology,' PrenticeHall, New Jersey, 1973.
8. J.S. Clunie, J.F. Goodman and P.C. Symons, Trans. Faraday Soc., 63, 754 (1967).
9. P. Mukerjee and K.J. Mysels, Critical Micelle Concentrations of Aqueous Surfactant Systems, NSRDS-NBS 36, U.S. Dept. of Commerce, Washington, D.C., 1971.
10. G.C. Kreshenk, p. 95 in 'Water', Vol.4 (Ed. F. Franks), Plenum Press, New York, 1975.
11. S.I. Ahmad and S. Friberg, J. Am. Chem. Soc., 94, 5196 (1972).
12. J.W. McBain and O.A. Hoffman, J. Phys. Colloid. Chem., 63, 39 (1949).
13. G.S. Hartley, Q. Rev. Chem. Soc., 2, 152 (1948).
14. F.M. Menger, Acc. Chem. Res., 12, 111 (1979).

15. E.H. Cordes, 'Reaction Kinetics in Micelles,' Plenum Press, New York, 1973.

16. J.H. Fendler and E.J. Fendler, 'Catalysis in Micellar and Macromolecular Systems,' Academic Press, New York, 1975.

17. C. Tanford, 'The Hydrophobic Effect,' Wiley Interscience, New York, 1973.

18. M.L. Das and F.L. Crane, Biochemistry, 3, 696 (1964).

19. M. Nemat-Gorgani and G.H. Dodd, Eur. J. Biochem., 74, 139 (1977).

20. J.H. Fendler, Acc. Chem. Res., 9, 153 (1976).

21. K. Shinoda, 'Solvent Properties of Surfactant Solutions,' Marcel Dekker, New York, 1967.

22. A.S. Kertes and H. Gutman, p. 193, 'Surface and Colloid Science,' Vol. 8 (Ed. E. Matijevic), Wiley, New Jersey, 1969.

23. See relevant sections in MTP International Review of Science, Series one, Physical Chemistry, Volume on Surface and Colloid Chemistry, (Ed. M. Kerker), Butterworths, London, 1973.

24. See relevant sections in MTP International Review of Science, Series Two, Physical Chemistry, Volume on Surface and Colloid Chemistry (Ed. M. Kerker), Butterworths, London, 1976.

25. E. Gonick, J. Colloid Sci., 1, 393 (1946).

26. H.F. Eicke and H. Christen, Helvit. Chim. Acta, 61, 2258 (1978).

27. P.S. Sheih, Ph.D. Thesis, Texas A & M University, 1976.

28. K. Kon-No and A. Kitahara, J. Colloid. Interface Sci., 35, 636 (1971).

29. J.H. Fendler, E.J. Fendler, R.T. Medary and O.A. El Seoud, J.C.S. Faraday I, 69, 280 (1973); J. Phys. Chem., 77, 1432 (1973).

30. O.A. El Seoud, E.J. Fendler, J.H. Fendler and R.T. Medary, J. Phys. Chem., 77, 1876 (1973).

31. N. Muller, J. Phys. Chem., 79, 287 (1975).

32. M.B. Mathews and E. Hirschhorn, J. Colloid Sci., 8, 86 (1953.).

33. K. Kon-No and A. Kitahara, J. Colloid. Interface Sci., 35, 409 (1971).

34. C.M. Aebi and J.R. Wiebush, J. Colloid Sci., 14, 161 (1959).

35. M. Wong, M. Gratzel and J.K. Thomas, J. Amer. Chem. Soc., 98, 2391 (1976).

36. M. Wong, J.K. Thomas and T. Nowak, J. Amer. Chem. Soc., 99, 4730 (1977).

37. F.M. Menger, G. Saito, G.V. Sanzero and J.R. Dodd, J. Amer. Chem. Soc., 97, 909 (1975).

38. F.M. Menger and G. Saito, J. Amer. Chem. Soc., 100, 4376 (1978).

39. F.M. Menger, J.A. Donohue and R.F. Williams, J. Amer. Chem. Soc., 95, 286 (1973).

40. J.H. Fendler and L.J. Liu, J. Amer. Chem. Soc., 97, 999 (1975).

41. J.H. Fendler, F. Nome and H.C. Van Woert, J. Amer. Chem. Soc., 96, 6745 (1974).

42. W. Hinze and J.H. Fendler, J. Chem. Soc., Dalton Trans., 238 (1975).

43. P.S. Sheih and J.H. Fendler, Results cited in Ref. 20.

44. F.P. Gentile, F. Ricci, F. Podo and P.E. Gna, Gazz. Chim. Ital., 106, 423 (1976).

45. M.A. Wells, Biochemistry, 13, 4937 (1974).

46. P. Elsworthy and D.S. McIntosh, J. Phys. Chem., 68, 3448 (1964).

47. J. Clifford, B.A. Pethica and E.G. Smith, p. 19 in 'Membrane Models and the Formation of Biological Membranes,' C.L. Bolis and B.A. Pethica, eds.) North Holland, Amsterdam (1968).

48. R. Wolf and P.L. Luisi, Biochem. Biophys. Res. Comm., 89, 209 (1979).

49. P.L. Luisi, F.J. Bonner, A. Pellegrini, P. Wiget and R.Wolf, Helvit. Chim. Acta, 62, 740 (1979).

50. See, for example, Chapter 10 on 'Detergency' in 'Surfactants and Interfacial Phenomena,' M.J. Rosen, Wiley Interscience, New York (1978).

51. K. Kon-No and A. Kitahara, J. Colloid. Interface Sci., 33, 124 (1970).

52. K. Kon-No and A. Kitahara, J. Colloid. Interface Sci., 33, 221 (1970).

53. K. Kon-No and A. Kitahara, J. Colloid. Interface Sci., 37, 469 (1971).

54. K. Kon-No and A. Kitahara, J. Colloid. Interface Sci., 41, 47, 86 (1972).

55. K. Shinoda and T. Ogava, J. Colloid. Interface Sci., 24, 56 (1967).

56. K. Shinoda and H. Saito, J. Colloid. Interface Sci., 26, 70 (1968).

57. K. Shinoda and H. Kunieda, J. Colloid. Interface Sci., 42, 381 (1973).

58. T.P. Hoar and J.H. Schulman, Nature, 152, 102 (1943).

59. D.O. Shah, V.K. Bansal, K. Chan and W.C. Hsieh, p. 293 in 'Improved Oil Recovery by Surfactant and Polymer Flooding,' (Eds. D.O. Shah and R.S. Schechter), Academic Press, New - york, 1977.

60. 'Microemulsions, Theory and Practice,' (Ed. L.M. Prince), Academic Press, New York, 1977.

61. J.H. Schulman and T.S. McRoberts, Trans. Farad. Soc., 42B, 165 (1946).

62. J.H. Schulman and D.P. Riley, J. Colloid Sci., 3, 383 (1948).

63. J.H. Schulman, T.S. McRoberts and D.P. Riley, J. Physiology, 107, 15 (1948).

64. J.H. Schulman and J.P. Friend, J. Colloid. Sci., 4, 457 (1949).

65. J.H. Schulman, R. Matalon and M. Cohen, Faraday Soc. Disc., 11, 117 (1951).

66. J.E. Bowcott and J.H. Schulman, Zeitschrift für Electrochemie Ber. Bunsenges. Physik. Chemie., 59, 283 (1955).

67. J.E. Schulman, W. Stoecknius and L.M. Prince, J. Phys. Chem., 63, 1677 (1959).

68. W. Stoecknius, J.H. Schulman and L.M. Prince, Koll. Zeil., 169, 170 (1960).

69. W.A. Zisman, J. Chem. Phys., 9, 789 (1941).

70. M.L. Robbins and V.K. LaMer, J. Phys. Chem., 62, 1291 (1958).

71. J.T. Davies and D.A. Haydon, Proceedings of the Second International Congress of Surface Activity, I, 417 (1957), cited in ref. 59.

72. L.M. Prince, J. Colloid. Interface Sci., 23, 165 (1967).

73. L.M. Prince, J. Colloid Interface Sci., 29, 216 (1969).

74. A.W. Adamson, J. Colloid. Interface Sci., 25, 261 (1969).

75. C.A. Miller and L.E. Scriven, J. Colloid. Interface Sci., 33, 360, 371 (1970).

76. S. Levine and K. Robinson, J. Phys. Chem., 76, 876 (1972).

77. E. Ruckenstein and J.C. Chi, J. Chem. Soc. Farad. Trans.II, 71, 1650 (1975).

78. M.L. Robbins, SPE-5836 at Improved Oil Recovery Symposium, Tulsa, U.S.A., March, 1976.

79. J.P. O'Connol and R.J. Bragman, p. 339 in 'Enhanced Oil Recovery by Surfactant and Polymer Flooding,' (Eds. D.O. Shah and R.S. Schechter), Academic Press, New York, 1977.

80. Many pertinent papers in 'Micellisation, Solubilisation Microemulsions,' Proc. Intl. Symposium (Ed. K.L. Mittal), Plenum Press, 1976.

81. D.O. Shah and R.M. Hamlin, Science, 171, 483 (1971).

82. D.O. Shah, A. Tamjeedi, J.W. Falco and R.D. Walker Jr., AIChE J., 18, 1116 (1972).

83. J.W. Falco, R.D. Walker Jr. and D.O. Shah, AIChE J., 20, 510 (1974).

84. D.O. Shah, R.D. Walker Jr., W.C. Hsieh, N.J. Shah, S.Dwivedi, J. Nelander, R. Pepinsky and D.W. Deamer, SPE-5815, presented at Improved Oil Recovery Symposium, Tulsa, U.S.A., March 1976.

85. D.O. Shah, Ann. New York Acad. Sci., 204, 125 (1973).

86. P.A. Winsor, Chem. Rev., 68, 1 (1968).

87. E. Sjoblom and S. Friberg, J. Colloid. Interface Sci., 67, 16 (1978).

88. H.L. Rosano, R.C. Peiser and A. Eydt, Rev. Francaise des Corps. Gras., 16, 249 (1969).

89. W. Gerbacia and H.L. Rosano, J. Colloid. Interface Sci., 44, 242 (1973).

90. A.A. Criniger, cited in p. 131, Ref. 60.

91. H.L. Rosano, J. Soc. Cosmetic Chem., 25, 609 (1974).

92. H.L. Rosano and A. Weiss, cited in p. 131, Ref. 60.

93. Kai-Li-Ko, cited in p. 131, Ref. 60.

94. L.M. Prince, J. Colloid. Interface Sci., 52, 182 (1975).

95. P. Ekwall, L. Mandell and K.J. Fontell, J. Colloid. Interface Sci., 33, 2156 (1970).

96. P.Ekwall, Acta Polytechnica Scandinavica Chem. Met. Ser., 74, I, 1 (1968), cited in Ref. 97.

97. S. Friberg, p. 133, in 'Microemulsion, Theory and Practice' (ed. by L.M. Prince), Academic Press, New York, 1977.

98. W.C. Tosch, C.C. Jones and A.W. Adamson, J. Colloid. Interface Sci., 31, 287 (1969).

99. L.M. Prince, J. Colloid. Interface Sci., 52, 182 (1975).

100. G.D. Smith, B.B. Garrett, S.L. Holt and R.E. Barden, J. Phys. Chem., 80, 1708 (1976); Inorg. Chem., 16, 558 (1977).

101. K. Letts and R.A. Mackay, Inorg. Chem., 14, 2990 (1975).

102. K. Letts and R.A. Mackay, Inorg. Chem., 14, 2993 (1975).

103. C.E. Jones and R.A. Mackay, J. Phys. Chem., 82, 63 (1978).

104. C.E. Jones, C.A. Jones and R.A. Mackay, J. Phys. Chem., 83, 805 (1979).

105. R.A. Mackay, K. Letts and C. Jones, p. 801 in 'Micellisation, Solubilisation, Microemulsions' (Ed. K.L. Mittal), Plenum Press, 1977.

106. A. Ray and G. Nemethy, J. Amer. Chem. Soc., 93, 6787 (1971).

107. A.A. Ribeiro and E.A. Dennis, J. Phys. Chem., 80, 1746 (1976).

CHAPTER II*

PHYSICAL STUDIES ON TRITON X-100 - ALCOHOL MICROEMULSIONS

---

*A paper based on work described in this chapter has been published:

C. Kumar and D. Balasubramanian, J. Colloid. Interface Sci., 69, 271 (1979).

In this chapter, we present evidence directed towards the establishment of the formation of microemulsions of the water-in-oil type in Triton X-100 : alcohol solutions in cyclohexane and an analysis at some length of the physical properties of the microemulsion structures formed. The reasons for the choice of the systems under study and the reasons for this study itself have already been discussed in the Introduction.

MATERIALS AND METHODS

The Triton X-100 used in the studies was purchased from CSIR Biochemicals Unit, New Delhi. The Triton X-100 used was cleared of any low boiling impurities by exposure to vacuum for 3 hours at 60-70°C. Cyclohexane used as solvent was freshly distilled after drying over sodium wire. The alcohols were purified by fractional distillation. Salts used were of AnalaR quality. Deionised, glass distilled water was used for making-up aqueous solutions.

To measure the maximum water uptake, different volumes of water were added to aliquots of a 20% w/v solution of the surfactant mixture in cyclohexane. The samples were then shaken to ensure mixing, allowed to stand for a few minutes, centrifuged at 800 rpm in a table-top centrifuge and then inspected for phase separation. The samples were tested for optical anisotropy by viewing the sample tube held between crossed polarisers against a strong light source. The ratio of Triton X-100 to alcohol (on a w/w basis) was varied in fixed steps. Based on

the results of the water uptake studies, Triton X-100 to alcohol ratios of 3:2 and 4:1 (w/w) were chosen for further investigation by other techniques.

Conductivity measurements were carried out by slowly adding a measured quantity of aqueous KCl solution to a fixed volume of the surfactant solution in cyclohexane. The conductivity of the system was measured after vigorous mixing after each addition. A Systronics 303 conductivity meter, standardized regularly against a 0.1 M KCl solution, was used for the measurements. The KCl solution, used as the indicating electrolyte in the titrations, had a specific conductance of 0.01286 mho cm^{-1}. All runs were repeated at least twice and averaged.

Viscosity measurements were made on freshly prepared samples. A Rheotest II rotating cup viscometer with measuring system N was used for the measurements. All measurements were carried out at a number of shear rates ranging from 1312 to 100 sec^{-1}. For non-Newtonian samples, the values of viscosity at a shear rate of 1312 sec^{-1} have been used for calculating the relative viscosities.

Light scattering studies were conducted using a Brice-Phoenix series 2000 light scattering photometer with appropriate accessories. A standard cylindrical cell and the narrow beam geometry were used, with the addition of water and mixing being carried out in the cell itself. To remove dust particles, all solutions were repeatedly filtered through a fine sintered glass funnel under nitrogen pressure. All glassware, including the

cells, were washed repeatedly in detergent solution, filtered water and then acetone. Drying was done in a clean box. Transmission electron microscopy was carried out on a Philips EM3 electron microscope. The samples were prepared by substituting the water (normally added to the surfactant solution to form microemulsions) with 1% KPT stain. The samples, with different volume percentages of the stain solution added, were applied on a carbon support film, air dried and examined in the electron microscope at 85 kV.

To ascertain the composition of the interfacial film the method used was similar to that of Gerbacia and Rosano (1). To a concentrated solution of Triton X-100 in cyclohexane was added a known volume of water to produce turbidity. Then the alcohol under consideration was added in small quantities from a microburette until the system becomes clear. A known volume of cyclohexane was now added to produce turbidity and the system again titrated to clarity with the alcohol and the process repeated. From these data, under the assumption that no alcohol is present in the solubilised water and that all the Triton X-100 is present at the (C_6H_{12}-H_2O) interface the composition of the interfacial film was calculated. All experiments were carried out at 30 $\pm$ 1°C.

RESULTS AND DISCUSSION

1. Water Uptake

Figure 2.1 gives the values of the amount of water taken up by Triton X-100 : alcohol solutions in cyclohexane. The total

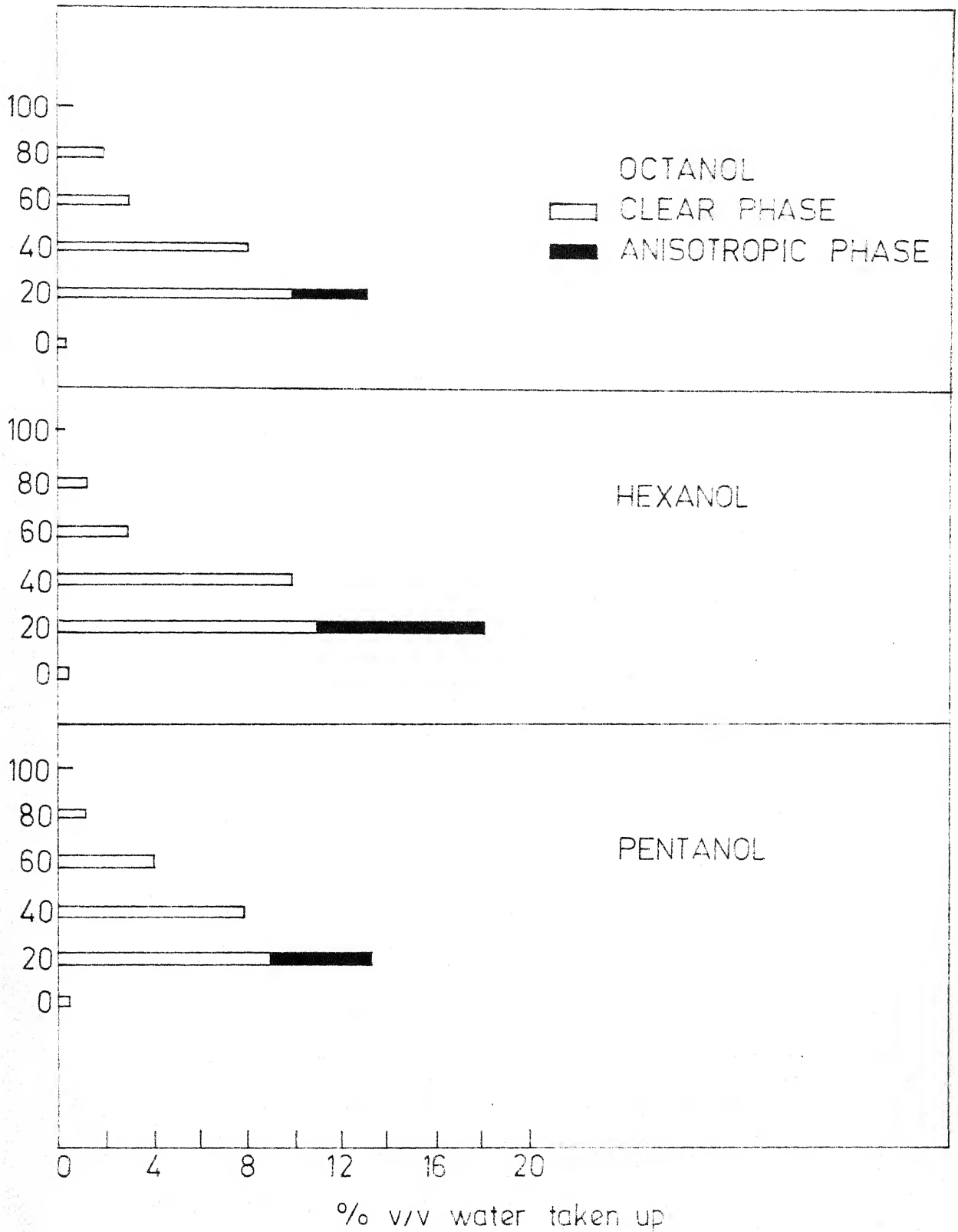


Fig. 2.1 Water uptake by a 20% w/v solution of Triton X100 - alcohol mixture in cyclohexane.

amount of (Triton X-100 + alcohol) in all cases was 20% w/v in cyclohexane.

At 30°C it was found that a 20% w/v solution of Triton X-100 alone in cyclohexane solubilises only about 0.1% v/v of water. This is a very small amount i.e., about 0.3 moles of water per mole of Triton X-100. This value is also comparable to those obtained by El Seoud (2) for Triton X-100 in CCl_4 solutions, who found that at less than 20% w/v concentrations in CCl_4, Triton X-100 solubilised less than 0.3 moles of water/mole Triton X-100. However, when the composition of the surfactant mixture is altered to 4:1 Triton X-100 : alcohol, we find that the amount of water taken up into clear, transparent solutions increases to 9%, 11% and 10% v/v (i.e., 16.7, 17.8 and 17.3 moles of water/mole of Triton X-100) for pentanol, hexanol and octanol respectively. At higher alcohol to Triton X-100 ratios the maximum amount of water taken up into clear solutions decreases. Hexanol seems to be somewhat more effective than pentanol and octanol in increasing water uptake by Triton X-100 solutions in cyclohexane. Another point of interest is the existence of a slightly turbid, anisotropic region before the onset of phase separation in the case of the 4:1 Triton X-100: alcohol mixtures after the clear isotropic region. This anisotropic region extends from 9 to 13%, 11 to 17% and 10 to 13% v/v water concentration in the pentanol, hexanol and octanol cases respectively.

Such behaviour is consistent with and understandable in terms of the past work on the water solubilisation efficiency

of surfactants by Shinoda and Kunieda (3) and Friberg et al.(4). Even though pure alcohol and pure Triton X-100 have very poor solubilising abilities by themselves, a mixture of the two, at an appropriate concentration ratio shows a vastly enhanced ability to solubilise water into cyclohexane. Shinoda and Kunieda (3) found that at any given temperature, for a given solvent and water, there exists an optimum hydrophile-lipophile balance (HLB) at which maximum solubilisation occurs. Also, for a given surfactant, maximum solubilisation occurs at a temperature close to its phase inversion temperature.

In our system, the main surfactant is Triton X-100. Triton X-100, due to its long ethylene oxide chain, is considerably hydrophilic and hence has a high phase inversion temperature. Hence, at 30°C, its solubilising ability is very low. But as an alcohol is added to it, the surfactant mixture becomes more hydrophobic. Its solubilising ability increases until an optimum alcohol concentration is reached, beyond which it decreases on further addition of alcohol. The enhancement of the efficiency of Triton X-100 for solubilisation of water into cyclohexane on a moles water solubilised/mole of Triton X-100 basis is even more significant than the variation seen in Figure 2.1, because the overall concentration of Triton X-100 decreases with increasing concentration of alcohol under the conditions of study.

It appears that the optimum Hydrophile-Lipophile balance occurs near an alcohol to Triton X-100 ratio of 1:4 for all the

three alcohols tested under our experimental conditions.

Water can be taken up into surfactant solutions in non-polar solvents to produce clear dispersions in two distinct ways: a) when the water is either molecularly dispersed in the solution, or b) is contained in nearly spherical reverse micelles or microemulsions with radii that are small in comparison with the wavelength of visible light. At the same time, the optical anisotropy of the turbid region in these systems can be explained if we assume the presence of ordered, highly asymmetric structures. Such structures can be either water cylinders or lamellae surrounded by surfactant films in which the surfactant molecules are oriented at the water-hydrocarbon interface with their hydrophobic part into the hydrocarbon continuous phase and the hydrophilic part into the aqueous phase. Such liquid crystalline assemblies are well known in literature in the case of the soap-alcohol-hydrocarbon water systems (5-7).

Though, as discussed in the Introduction, Friberg has pointed out that such liquid crystalline structures in similar surfactant systems fall in the multiphasic region of the phase diagram (Fig. 1.3), we have treated the liquid crystalline, anisotropic region as if it were a single phase. This is due to the high phase stability of the system in the region. Storage of samples in the liquid crystalline region for months did not lead to any phase separation. Hence, we have treated samples in this region as if made of entirely a single homogeneous phase in our further experimental studies.

Another point of interest is that the high values of water solubilised/mole of Triton X-100 (more than 15 moles H_2O/mole Triton X-100) suggest that all the water in the system will not be in a form bound to the surfactant. It is more likely that discrete pools of free, unbound water (contained by a surfactant film) exist in the system.

2. Conductivity Studies

Figure 2.2 gives the variation in the conductivity of a 20% w/v solution of a 3:2 (w/w) mixture of Triton X-100 and alcohol in cyclohexane as aqueous KCl is added to it. The initial addition of (upto 5% v/v) KCl solution does not lead to any significant increase in the specific conductivity of the solution. The specific conductivity values start rising rapidly prior to the phase separation point and continue rising even beyond phase separation.

Now, if the added electrolyte solution was molecularly dispersed through the bulk of the solvent, it would lead to a significant increase in the number of carriers available for electrical conduction and we would expect the specific conductance to rise steeply even for additions of small volumes, say 1% v/v, of the electrolyte solution. The absence of such behaviour in our systems indicates that the aqueous phase is not molecularly dispersed.

On the other hand, the insensitivity of specific conductance to added electrolyte solution can be explained if we assume

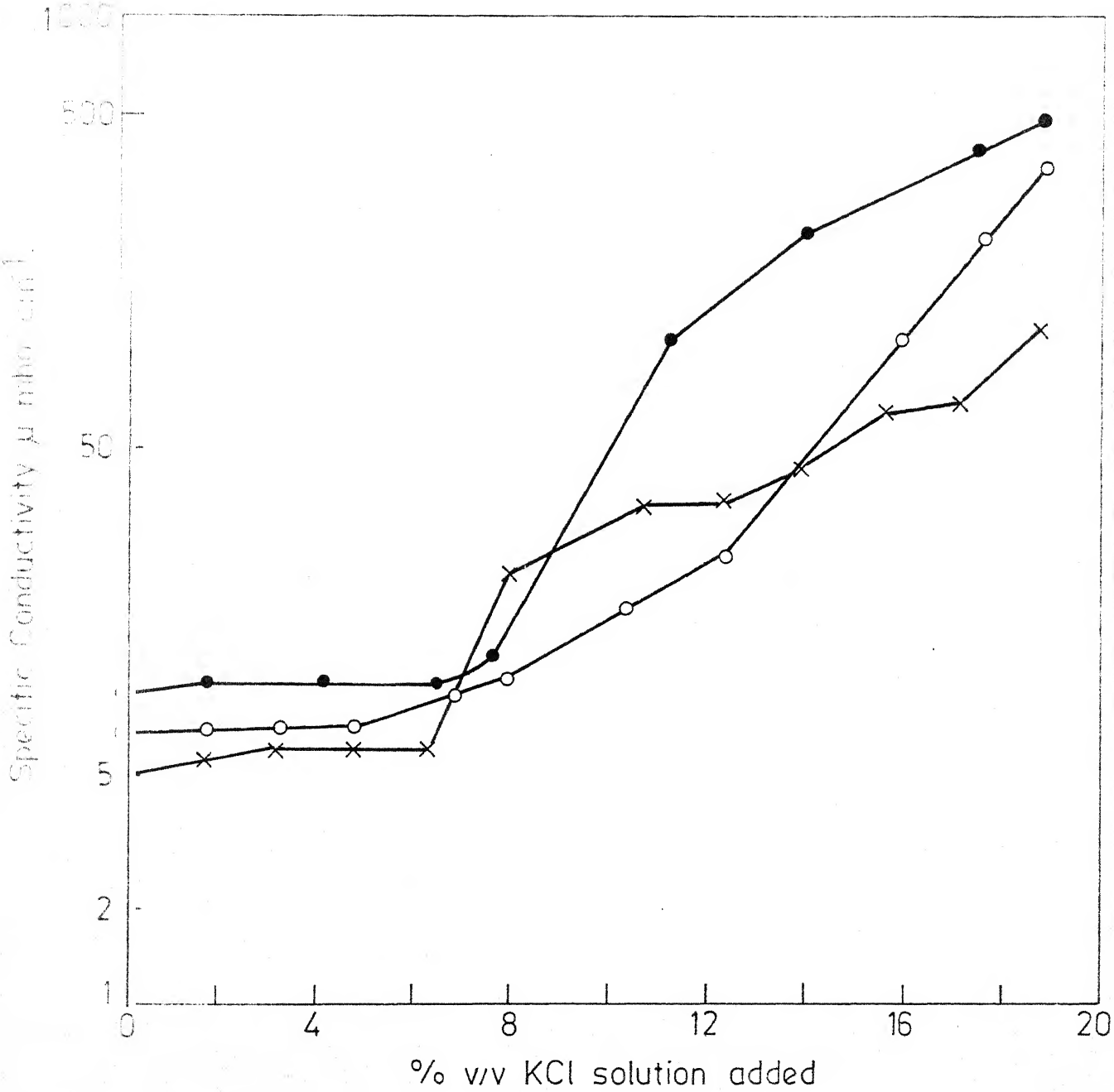


Fig. 2.2 Variation in specific conductivity of 20% w/v solution of Triton X 100 - Alcohol (3:2 w/w) in cyclohexane with amou of aqueous KCl added.
● - Pentanol, o - Octanol, × - Hexanol.

the aqueous electrolyte solution to be contained in specific water pools surrounded and stabilised by a surfactant film. Under these conditions the interfacial surfactant film and the continuous organic phase between such water pools would form a significant barrier to conduction. Conduction can take place only through the contact of specific water pools with the electrodes. Hence,we conclude that the added electrolyte solution is contained in discrete water pools that are formed in the interior of these microemulsions. Such conductivity measurements have been used in the past to distinguish between solubilisation that occurs through microemulsion formation and that through molecular dispersion (8,9). The increase in conductior just before phase separation in these systems can be taken to be indicative of the enlargement of the size of the water pools The conductivity is high after the phase separation point because there is no significant barrier to conduction in the stable macroemulsion formed. It is also to be noted that the conductivity behaviour is similar for all the three alcohols used as cosurfactants.

Figure 2.3 shows the variation in the specific conductan of a 20% w/v solution in cyclohexane of a 4:1 Triton X-100 : alcohol mixture. In these cases, we find that for the initial additions of KCl the specific conductance does not change from the organic phase values, then increases slowly at the point where the clear to turbid (or isotropic to anisotropic) phase transition occurs, then falls to an intermediate value and

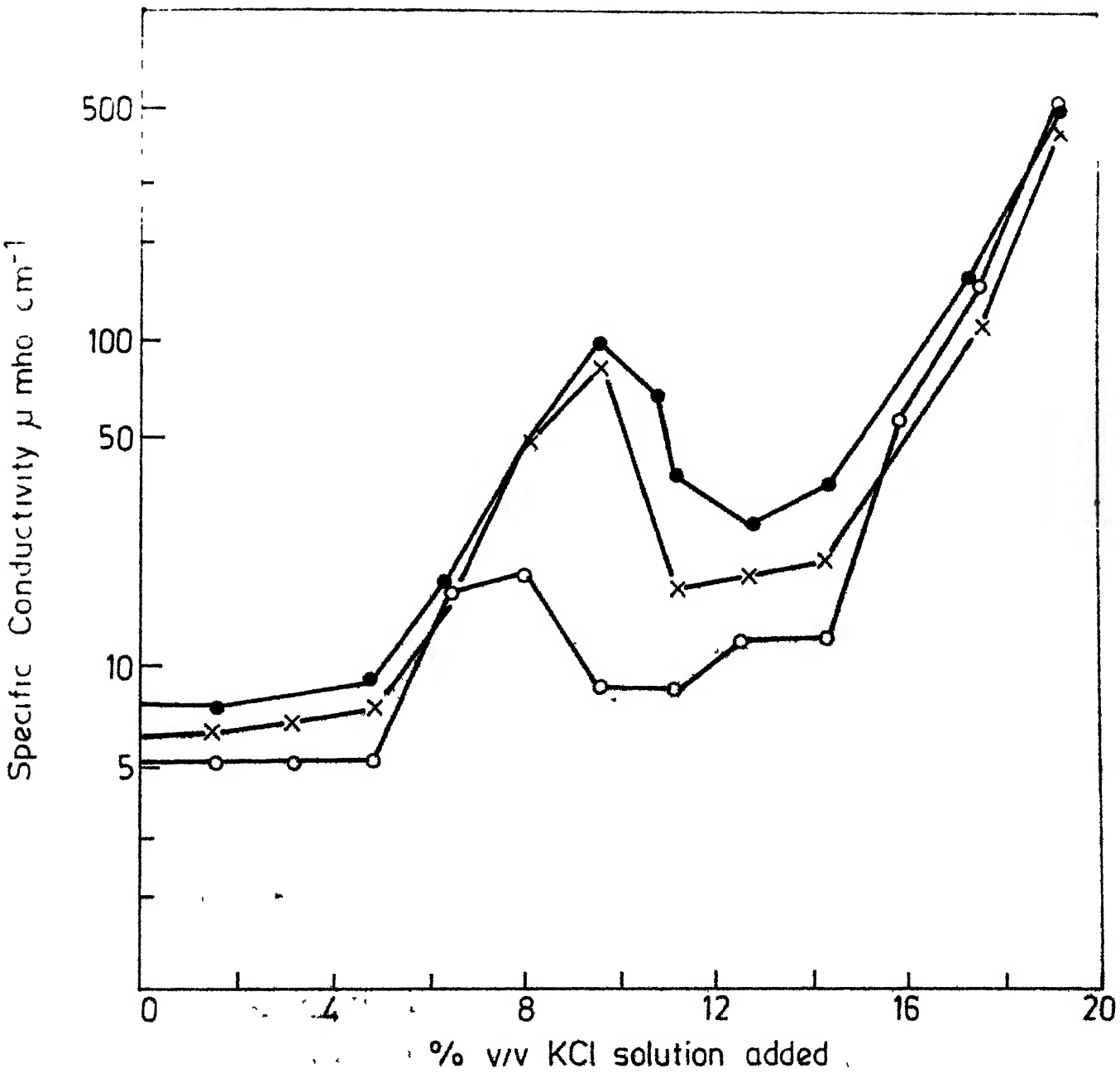


Fig. 2.3 Variation in specific conductivity of 20% w/v solution of Triton X-100 - Alcohol (4:1 w/w) in cyclohexane with amount of aqueous KCl added.
● - Pentanol, o - Octanol, x - Hexanol.

rises further again upto and beyond the phase separation point. While for the initial and final regions the same reasoning as in the 3:2 Triton X-100 : alcohol case will hold, the fall in specific conductivity in the earlier part of the aniosotropic region is a point of interest. If we assume that the aniso-tropic region contains water-surfactant cylinders or lamellae, the continuous aqueous channels found in these structures would be expected to make conduction easier than in the isotropic region. But this factor is opposed by the increased macro-scopic, and perhaps microscopic, viscosity of the system due to the presence of large asymmetric structures. This increase in viscosity would reduce the mobility of the water cylinders as a whole and of the conducting ions through the water channel. Initially in the anisotropic region this viscosity effect over-rides the effect of longer water channels. Later as more water is added, the ease of conduction increases and the specific conductance rises due to the greater size of the aqueous channels.

3. Viscosity Studies

In Figure 2.4 are shown the variation in the relative viscosities of the 3:2 w/w Triton X-100 : alcohol mixtures in cyclohexane for different alcohols as a function of added water. Similar viscosity data for a 4:1 w/w Triton X-100 to alcohol ratio are presented in Fig. 2.5.

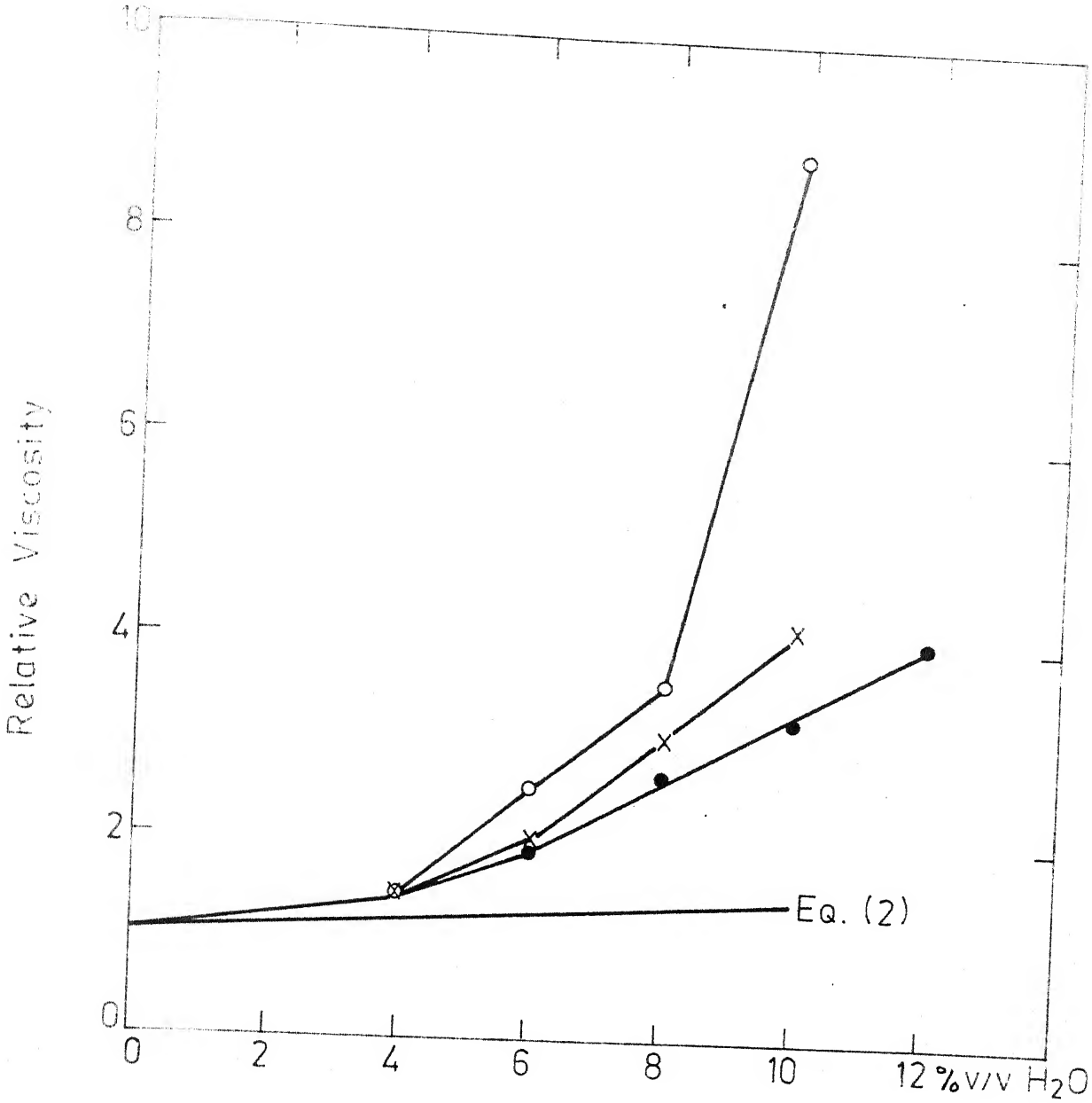


Fig. 2.4 Variation of relative viscosity of 20% w/v solution in cyclohexane of Triton X 100 - Alcohol (3:2 w/w) with amount of water added.

- ● - Pentanol
- x - Hexanol
- o - Octanol

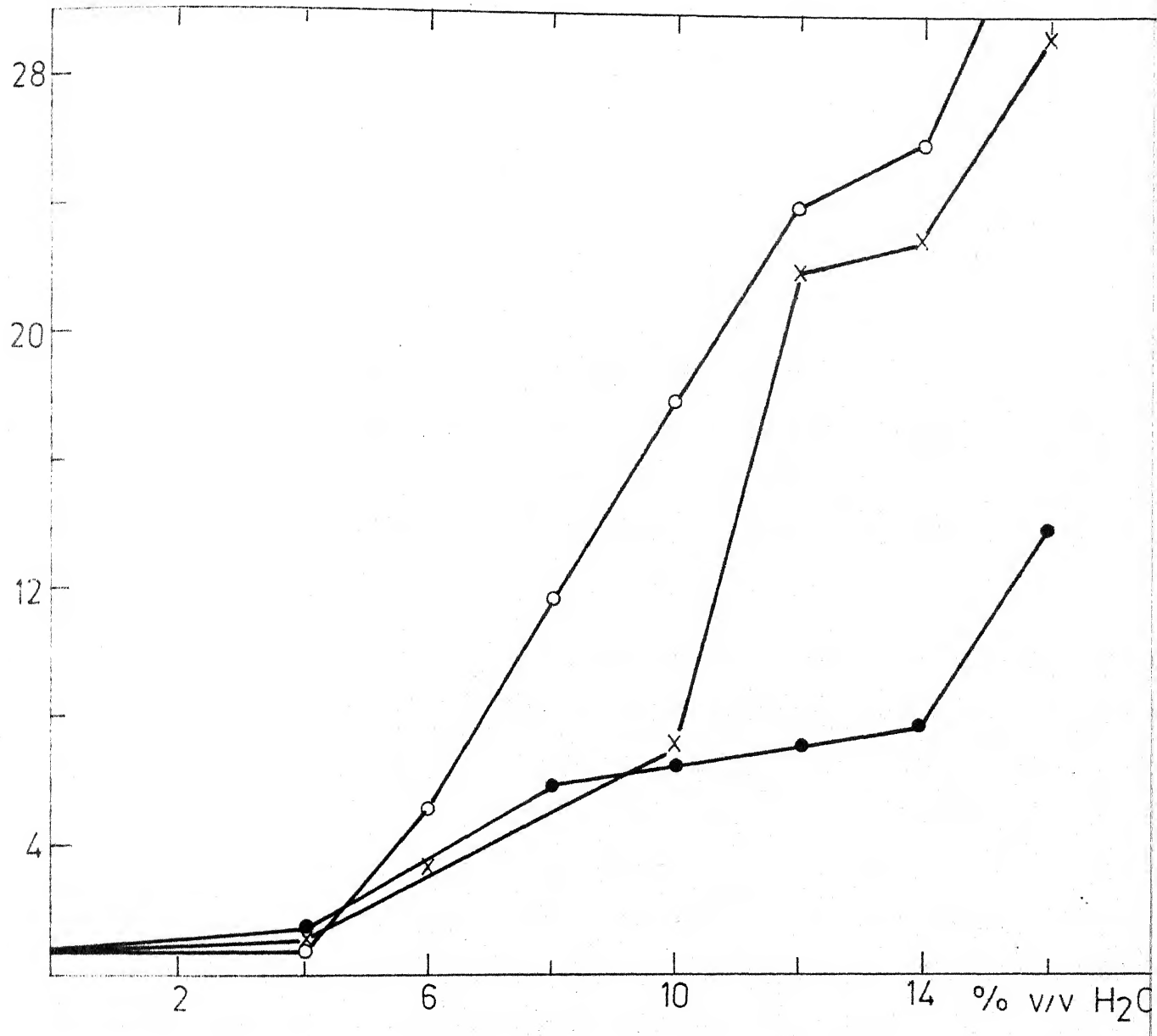


Fig. 2.5 Variation of relative viscosity of 20% w/v solution in cyclo hexane of Triton X 100 - Alcohol (4:1 w/w) with amount of water added.

- ● - Pentanol.
- x - Hexanol
- o - Octanol

The qualitative features of these viscosity curves are consistent with the conclusions based on conductivity and anisotropy data. For the 3:2 Triton X-100 : alcohol mixture, we see from Figure 2.4 that as water is added the relative viscosity of the system increases slowly in the initial portion, and later somewhat steeply until after phase separation occurs. This would be the natural consequence of introducing water as a dispersed phase into the initially homogeneous surfactant solution. All solutions showed Newtonian behaviour in this composition of the system, suggesting that the dispersed phase is present in highly symmetric, i.e. spherical-shaped, droplets.

In the 4:1 case, it can be seen from Fig. 2.5 that the initial part of the viscosity curves are very similar to the 3:2 case. Additional features seen are that the relative viscosity increases to very high values as the phase transition from isotropic to anisotropic phase occurs. In the anisotropic phase, all solutions tested displayed strongly non-Newtonian viscosity behaviour. Figure 2.6 shows the shear rate dependence of the viscosity values of one such representative solution. This strongly non-Newtonian behaviour exhibited by the anisotropic phase with all the three alcohols can be interpreted as due to the presence of highly asymmetric structures such as cylinders of lamellae in the system under these conditions (10).

Another interesting property shown by the 4:1 system is Thixotropy. At any given shear rate, anisotropic phases of the 4:1 system which have been allowed to stand for 24 hours gave

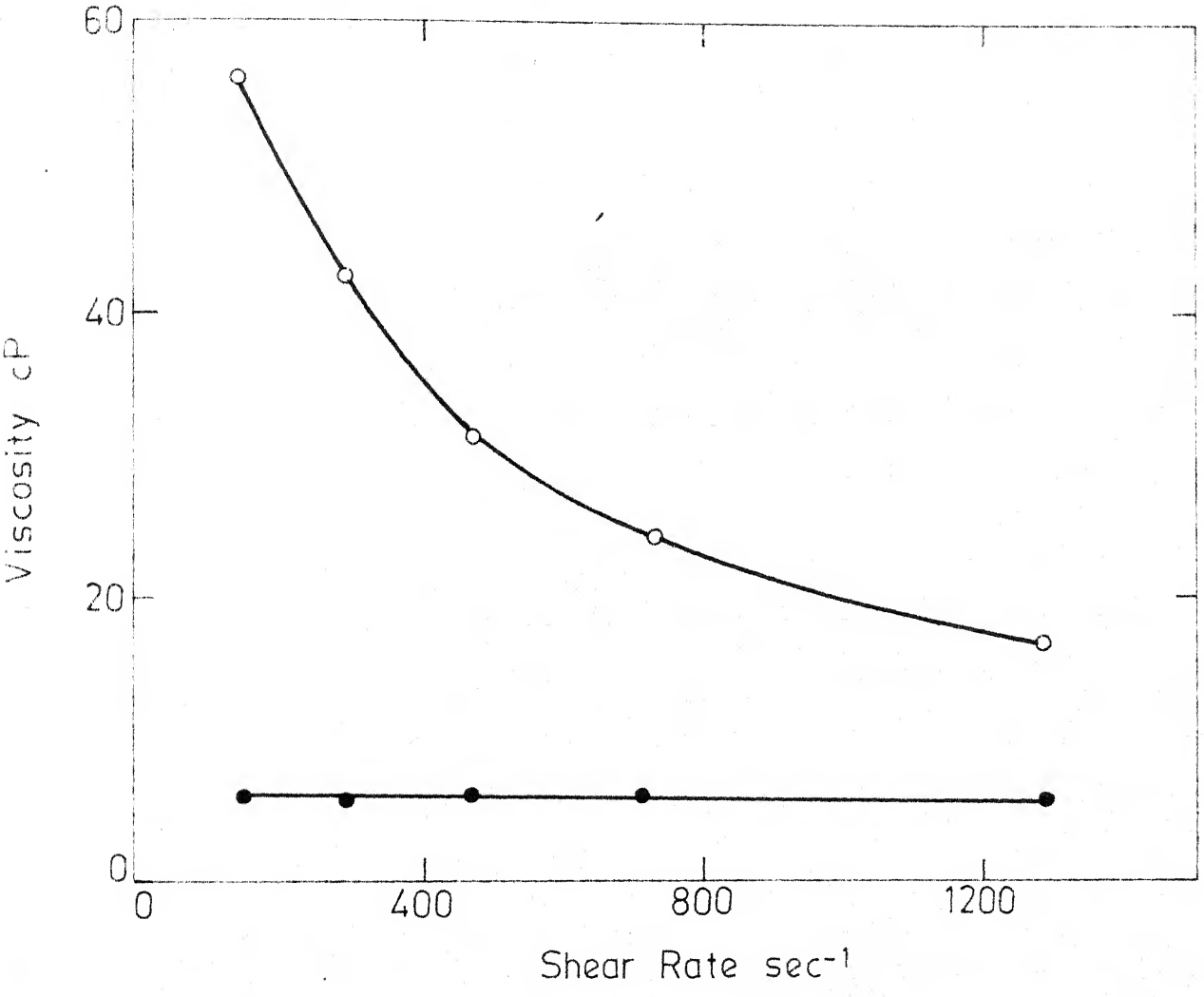


Fig. 2.6 Shear rate dependance of viscosity of 20% w/v solution in cyclohexane of Triton X 100 - hexanol (4:1 w/w).
Water added: ● — 9% v/v,
o — 11% v/v.

viscosity values that were initially lower than those of freshly prepared solutions. Under shear these anomalously low viscosities increased slowly over about 5 minutes to values close to those of freshly shaken solutions. Such behaviour has been noticed in the past in the lamellar phase of the system, potassium oleate-hexanol-hexadecane-water and has been interpreted to mean that the water-surfactant lamellae become ordered with respect to each other on standing, leading to low viscosity values (11). Such ordering is destroyed on shaking or upon the application of shear and the system gives viscosity values close to freshly prepared solutions in which there is no such ordering.

Our attempts to quantitatively analyse the relative viscosity values for the 4:1 and 3:2 isotropic phases failed as none of the available expressions for the dependence of relative viscosity upon the volume fraction of dispersed phase seem to fit the observed data well.

For very dilute suspensions of spheres in a liquid, Einstein has calculated the relative viscosity to be

$$\eta_r = 1 + 2.5\,\phi \qquad \ldots (1)$$

where η_r is the relative viscosity and ϕ is the volume fraction of dispersed phase (12). For high values of ϕ, in cases where the dispersed phase is present in the form of uniform spheres, Roscoe gives the expression (13):

$$\eta_r = (1 - 3.5\,\phi)^{-2.5} \qquad \ldots (2)$$

In Figure 2.4 we have shown the values of the relative viscosity calculated using Eqn. (2) with water as the dispersed phase and the surfactant solution as the continuous phase. The fact that the measured relative viscosities are significantly higher than the relative viscosities calculated using this expression would seem to indicate that the effective hydrodynamic volume of the water spheres is greater than the volume of water present alone. This difference could be ascribed to (a) the presence of the surfactant film at the water-cyclohexane interface; (b) the binding of solvent hydrocarbon to the surfactant hydrocarbon chains. Both of these factors will lead to increased effective volumes of the water spheres. The discrepancy between the calculated and experimental relative viscosities increases markedly at the phase transition point in the 4:1 case, consistent with the spherical - lamellar (or cylindrical) shape change of the water pools at higher concentrations of water.

Another factor of importance in relative viscosity calculations is the size of the dispersed phase droplets. It has been shown that at a given value of ϕ, the relative viscosity increases as the particle radius decreases and also, the relative viscosity increases with increasing attraction between the dispersed phase droplets (14-16). Since it is reasonable to expect that both the size of the water pool and the nature of

the interfacial film, and hence interdroplet attraction, would change with increasing water content in our system, a complex interplay of these factors could be the reason for the large differences between the calculated and theoretical values of relative viscosity in these systems.

4. Light Scattering Studies

In Figs. 2.7 and 2.8 are shown the light scattering behaviour of the 3:2 and 4:1 Triton X-100 : pentanol mixture solutions in C_6H_{12}, 20% w/v, as a function of added water. The parameters whose variation is shown are the ratio of light scattered at 90° to that at 0° (I_{90}/I_0), and the dissymmetry ratio, that is the ratio of light scattered at 45° to that scattered at 135°. These parameters behave in a qualitatively similar fashion in the case of hexanol - Triton X-100 and octanol-Triton X-100 also with appropriate shifts in the water concentration value at which discontinuities in these plots occur.

Since in our system the concentration of water and surfactants used is rather high, the light scattering parameters are bound to be affected by multiple scattering. Since the available theories of light scattering quantitatively discuss only the light scattered by particles in dilute solution (17), we can utilise light scattering as only a qualitative tool.

It can be seen from Figs. 2.7 and 2.8, that the ratio I_{90}/I_0 rises very slowly with increasing water concentration initially in both the 4:1 and 3:2 cases. In the 3:2 case, this

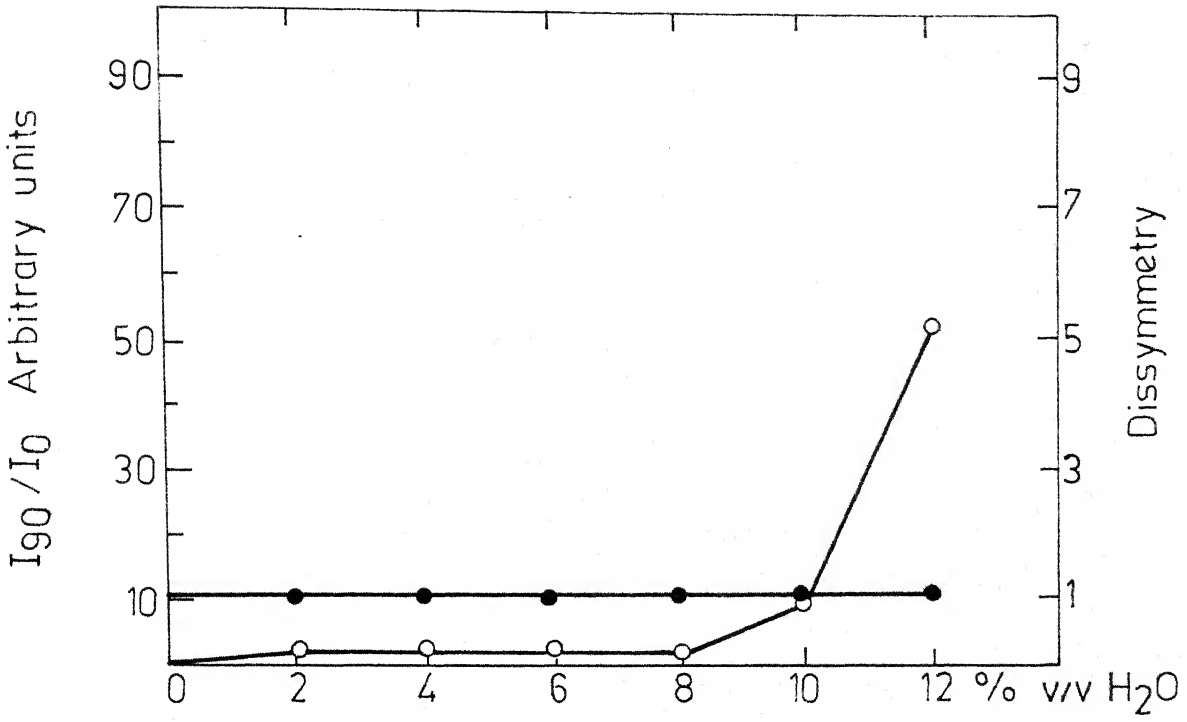


Fig. 2.7

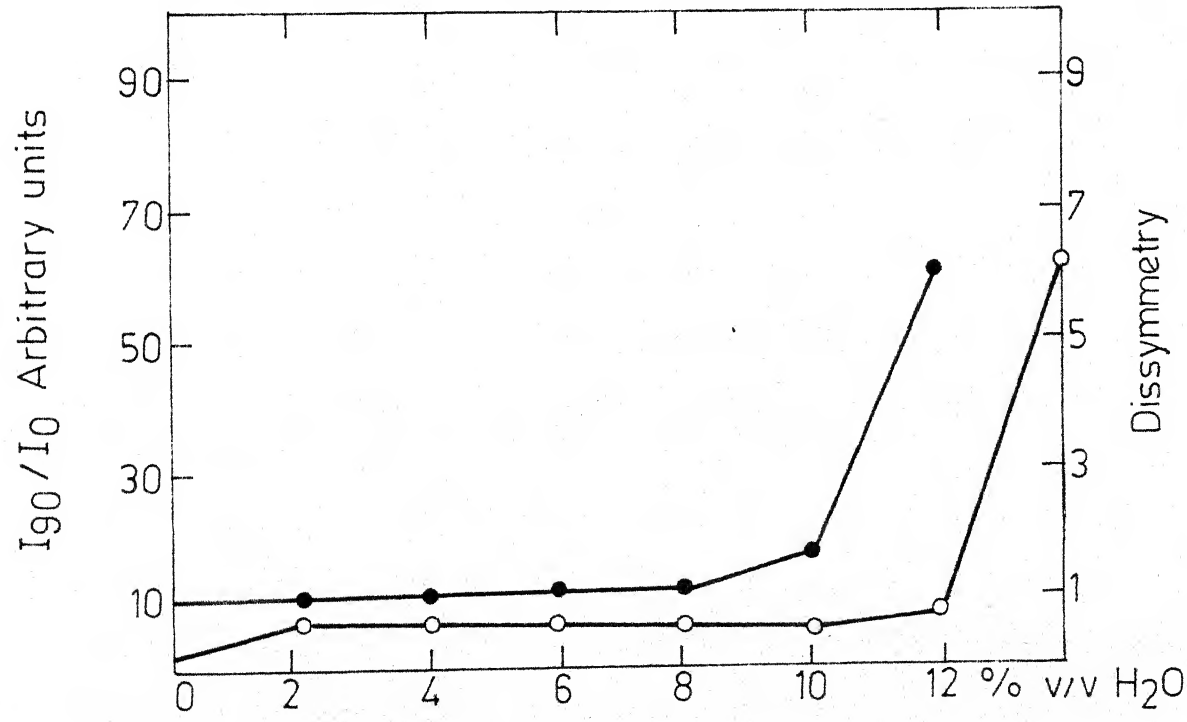


Fig. 2.8

Variation in light scattered at 90° (o—o—o) and dissymmetry (●—●—●) of 20% w/v solution in cyclohexane of Triton X 100 - pentanol (3:2 w/w, Fig. 2.7; 4:1 w/w, Fig. 2.8)

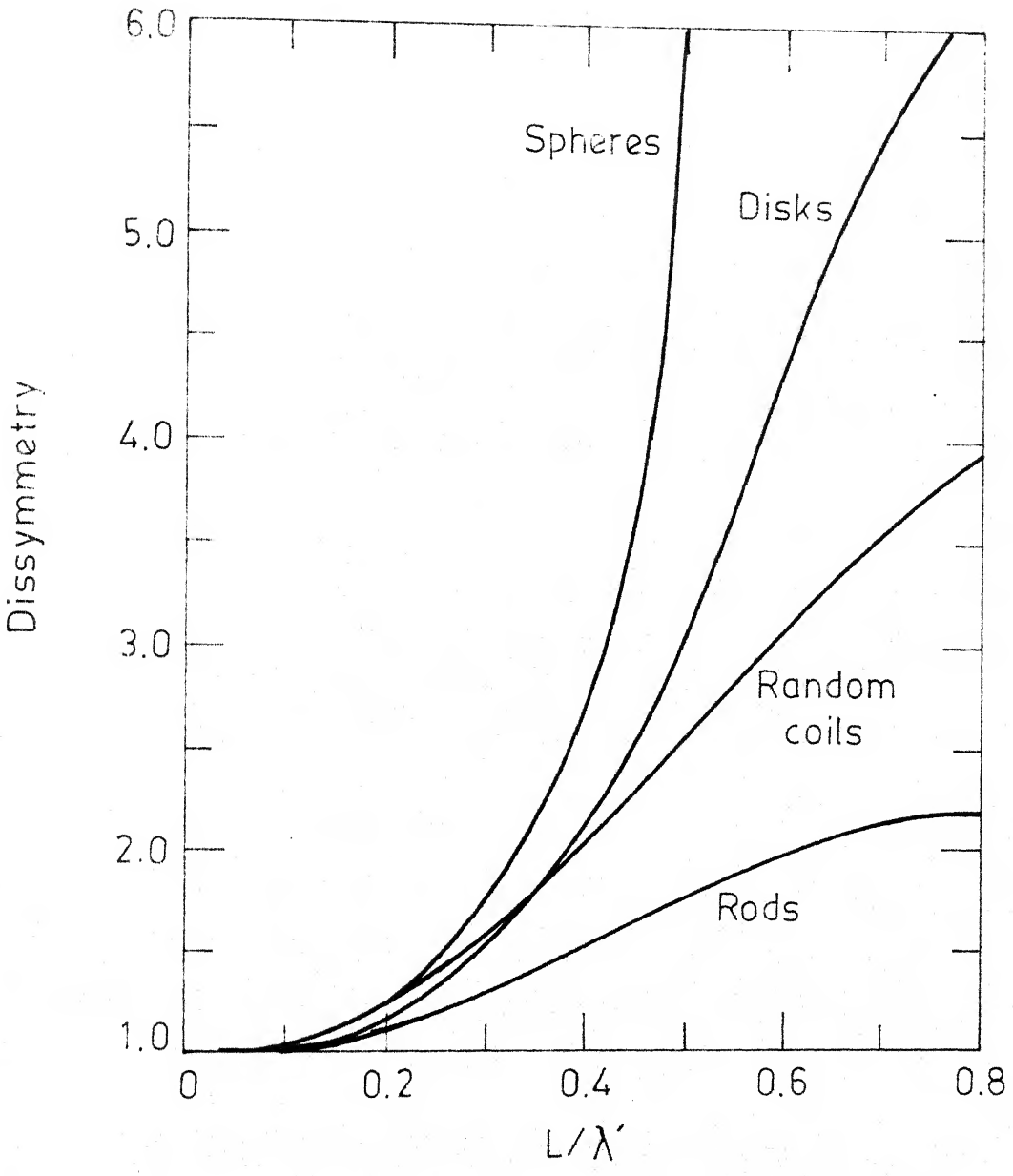


Fig. 2.9 Variation of dissymmetry with the radius of gyration of the scattering particles for different shapes.

disc-like structures. Since we have already ruled out the presence of spherical scattering centres in this region on the basis of anisotropy and viscosity data, we conclude that the anisotropic region consists of disc-like water-surfactant structures. For such structures, the radius of gyration would be around 2000 Å, as judged from the dissymmetry values.

It should also be mentioned that in the anisotropic region of 4:1 Triton X-100 : alcohol mixtures we found the reproducibility of light scattering measurements to be rather low, with run to run variations of the order of 15% of the average. Such large variations may perhaps have their origin in the variable ordering of the lamellar structures in the anisotropic region with respect to each other, as already pointed out in the discussion of their rheological properties.

5. Electron Microscopy

Schulman and coworkers (19,20) had utilised negative staining to obtain electron micrographs of some microemulsion systems before. Later, freeze etching has been utilised to look at liquid crystalline phases (9).

We have found that water-in-oil microemulsions can be investigated by positive staining. If the stain is included in the aqueous phase, it will be localised within the core of the microemulsion and liquid crystalline phases. In the case of Triton X-100 microemulsions, we can expect also the polyethylene oxide chain of the surfactant to be stained. Hence, if we

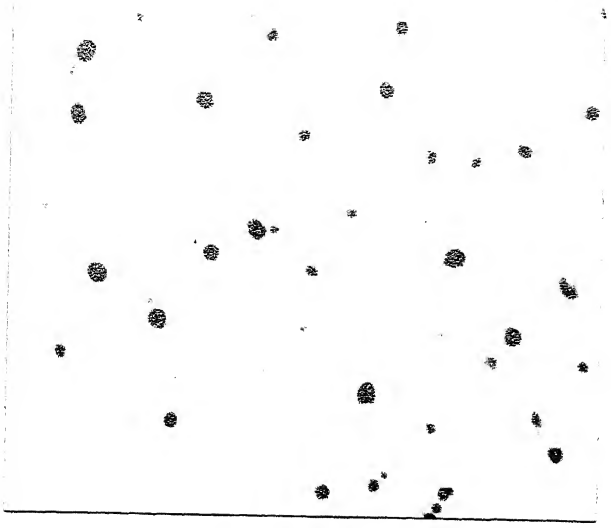


Fig. 2.10. Electron Micrograph of positively stained Triton X-100 - hexanol 4:1 system.
water concn. = 8% v/v; magnification = 50000.

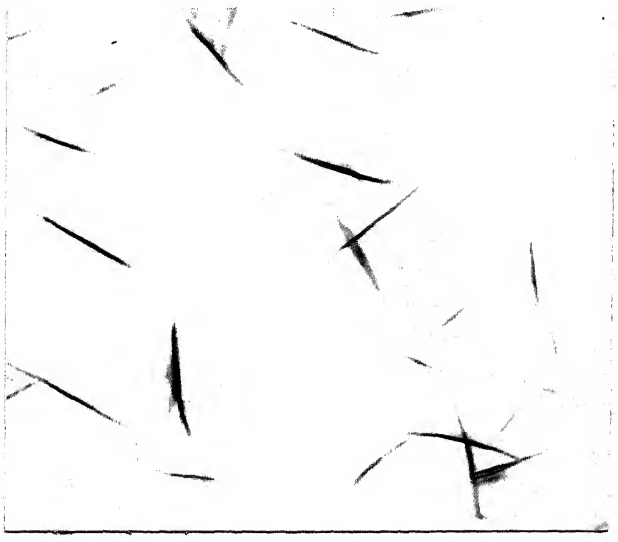


Fig. 2.11. Electron Micrograph of positively stained Triton X-100 - hexanol 4:1 system.
water concn. = 13% v/v; magnification = 100000.

include the stain in the aqueous phase of, say, a spherical microemulsion, the electron micrograph will show dark, electron impermeable circles on a white background. The background, (i.e. the bulk, organic phase) will be electron transparent because the ionic stain will not dissolve or partition into the organic phase. The radius of the circle, in the above example, should correspond to the sum of the radius of the aqueous core plus the thickness of the polyoxyethylene shell.

Such positively stained electron micrographs were obtained on a number of samples of the 4:1 and 3:2 Triton X-100 : hexanol samples between 1-16% v/v water content. Two such micrographs are presented in Figs. 2.10 and 2.11.

Upto 3% v/v water content, no electron micrographs could be obtained due to poor contrast. Above this, in the spherical microemulsion region, micrographs similar to Fig. 2.10 were obtained. A study of these micrographs showed that the aqueous cores of the spherical microemulsions were not homogeneous in size, but had a wide distribution. As the water content increased, the size distribution shifted towards larger dimensions. At 4% v/v water concentration, for both the 3:2 and 4:1 systems microemulsions cores in the range 100-150 Å radius predominate while at 8% water concentration, 200-330 Å radius becomes common.

In the 4:1 system at water concentrations corresponding to the lamellar phase, long thin lines are seen in the electron micrograph, as in Fig. 2.11. These correspond to cross-sections through the aqueous phase of the liquid crystals. The electron

micrographs show that the aqueous phase in the bilayer is contained in thin lamellae circa 2000 Å long and about 50-70 Å thick. There was no evident effect of changing water concentration on the dimensions of these needle like cross sections as long as the water concentration remained within the liquid crystalline range.

These EM observations support the conclusions arrived at from other studies discussed so far.

INTERFACIAL COMPOSITION

In Fig. 2.12, we show a typical plot of the amount of alcohol needed to produce clear microemulsions from a mixture of a fixed amount of Triton X-100 and water and varying amounts of cyclohexane against the volume of cyclohexane present.

Since the alcohol can be present: (a) as a solution in dispersed water, (b) at the interface, and (c) in the cyclohexane continuous phase, we can write:

$$\text{Total alcohol} = \text{alcohol } (H_2O) + \text{alcohol (interface)} + \text{alcohol (cyclohexane)}.$$

Hence, the intercept at the y-axis in Fig. 2.12 corresponds under the given conditions to alcohol at the interface (if we neglect the alcohol dissolved in water) while the slope of the line corresponds to the concentration of alcohol in the bulk phase. From these data the equilibrium constant for the process,

$$\text{alcohol (cyclohexane)} \rightleftharpoons \text{alcohol (interface)}$$

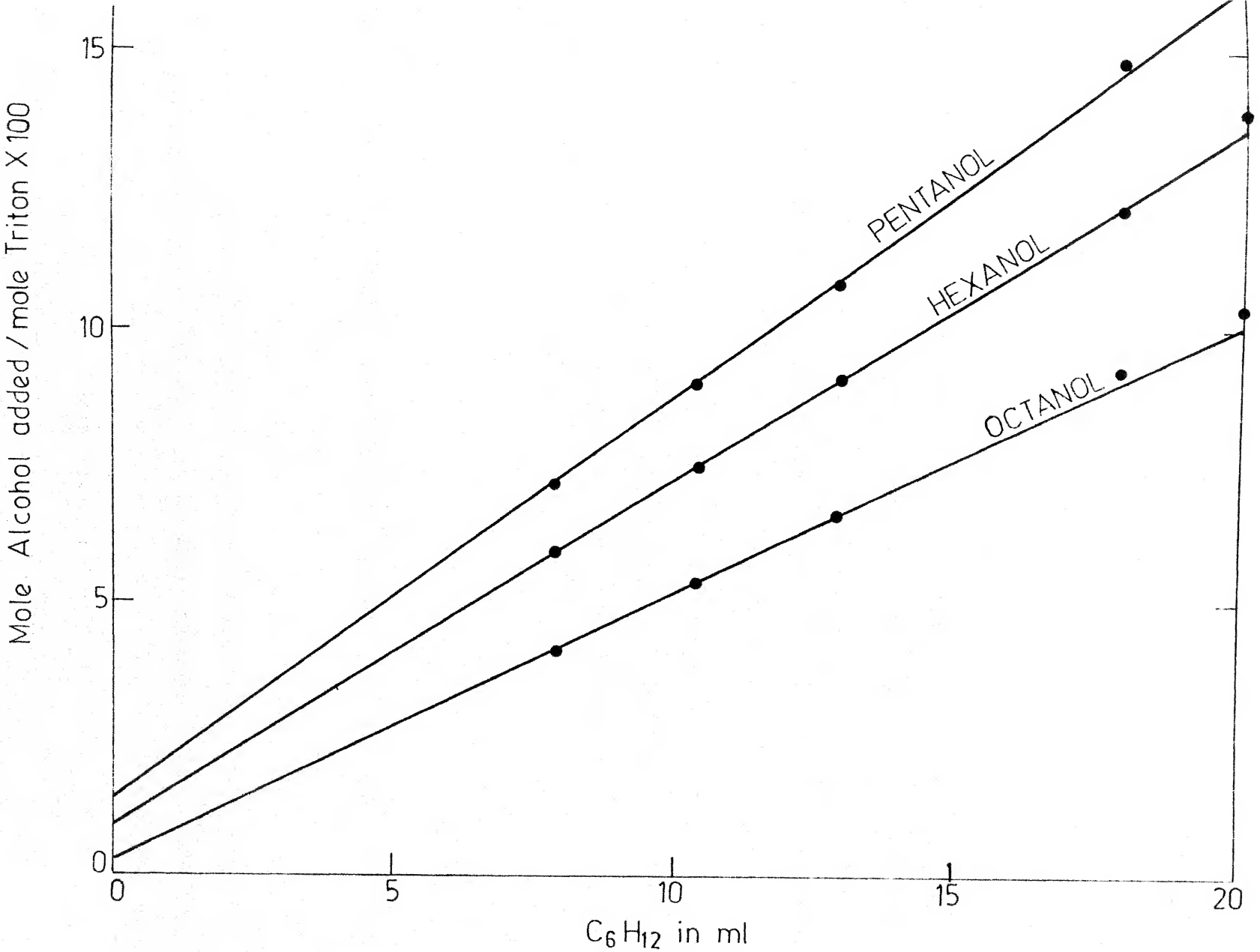


Fig. 2.12 Estimation of fraction of Alcohol present at interface.

can be calculated and also the free energy to transfer of one mole of alcohol from the bulk phase to the interface. These values are tabulated in Table 2.1.

It will be seen from Table 2.1 that the free energies of transfer to the interface of the alcohols are small and negative. Their magnitude decreases with increasing chain-length for the three alcohols tested. It can be speculated that these differences in the magnitude of ΔG values for transfer to interface are essentially reflective of the entropy effects; the ΔH of transfer to interface of the three alcohols may not differ much, since transfer to the interface does not significantly change the hydrocarbon environment of the alkyl part of the alcohols while the head group that is transferred from the hydrocarbon to the aqueous phase is identical in the three alcohols. Then, from

$$\Delta G = \Delta H - T\Delta S,$$

we infer that the change in entropy due to the transfer of the alcohol to the interface varies in the order pentanol $>$ hexanol $>$ octanol, which is the order intuitively expected if size disparity between the alcohol and the Triton X-100 alkyl chain is a major factor in causing the entropy change. Such a scheme would also be consistent with Schulman's discussion of the role of the alcohol in the formation of the microemulsions, as already described in the Introduction (19).

Table 4.1

	Alcohol	Mole alcohol/ Mole Triton X-100[a]	Mole fraction at Interface of alcohol	Mole alcohol/ Mole cyclo-hexane	Mole fraction of alcohol in contin. phase	ΔG_{trans} cal/mole[b]
1	Pentanol	0.135	0.1190	0.0239	0.0234	-980.4
2	Hexanol	0.080	0.0748	0.0209	0.0204	-775.7
3	Octanol	0.030	0.0293	0.0155	0.0153	-391.3

a. Water to Triton X-100 ratio = 1:5 w/w.

b. $\pm$ 3%.

Another point of interest is that the alcohol : surfactant ratio in the microemulsion interface is lower for Triton X-100 in comparison with many other ionic surfactants(1,21). This is probably due to the lower hydrophilicity of Triton X-100 in comparison with ionic surfactants like potassium oleate or sodium dodecyl sulfate.

CONCLUSIONS

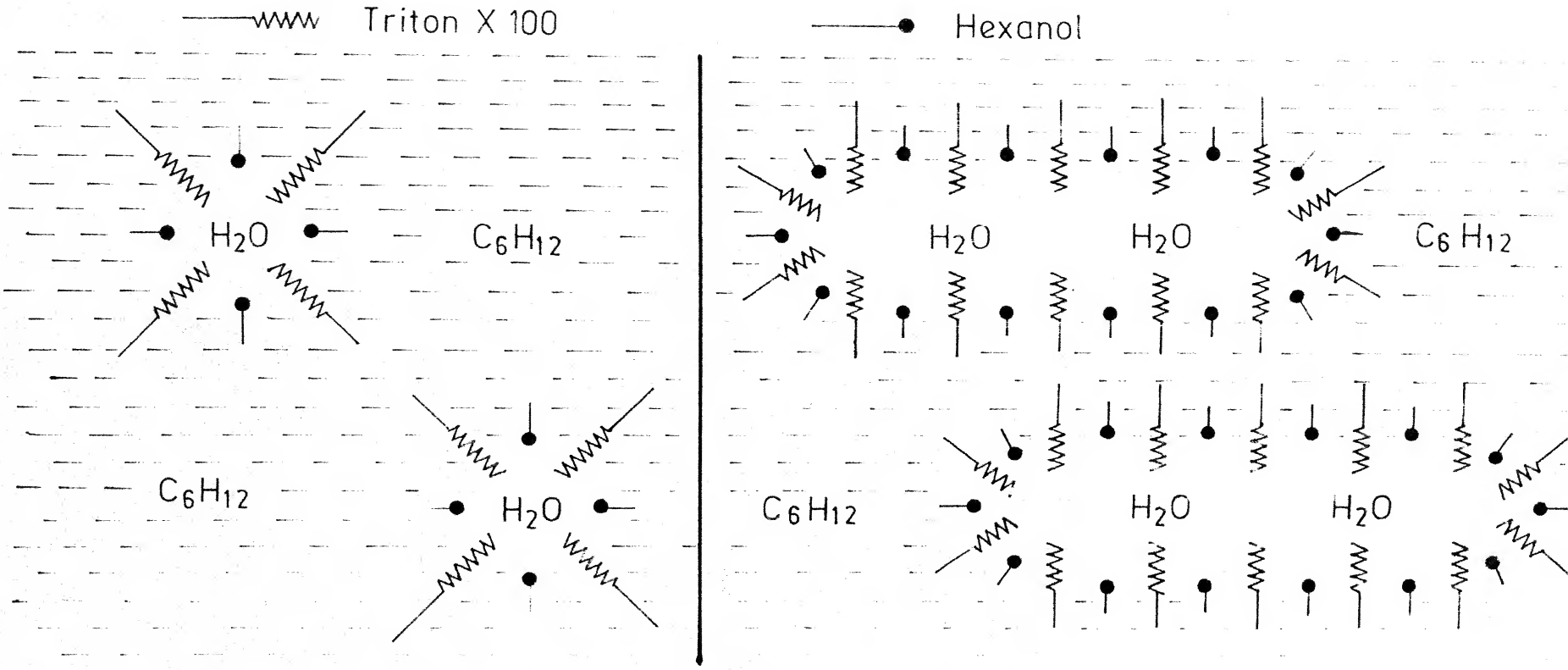
In short, the experimental results presented in this chapter have led us to the following conclusions:

(1) Water uptake by Triton X-100 solutions in cyclohexane is enhanced by the addition of alcohols.

(2) A 3:2 w/w mixture of Triton X-100 : alcohol mixture (20%, w/v) in cyclohexane solubilises respectively 8, 9 and 6% v/v of water when the alcohol is pentanol, hexanol or octanol into clear microemulsions.

(3) A 4:1 w/w mixture of Triton X-100: alcohol mixture (20%, w/v) in cyclohexane solubilises respectively 9, 11 and 10% v/v of water into clear microemulsions and a further 4, 6 and 3% of water into a turbid liquid crystalline phase respectively when the alcohol is pentanol, hexanol or octanol.

(4) Optical anisotropy, conductivity, viscosity and light scattering measurements as a function of water concentration show that the clear microemulsions contain dispersed water in small spherical droplets which are surrounded by a surfactant interfacial layer while the turbid phase contains large, ordered



LOW WATER CONCETRATION
Spherical microemulsion
$R_G < 300\,Å$

HIGHER WATER CONCENTRATION
Lamellar liquid crystals
R_G circa 2000 Å

Fig. 2.13

water-surfactant lamellae. The possible structures are shown schematically in Fig. 2.11.

(5) Measurement of alcohol requirements for microemulsion production in this system shows that only a small fraction of the alcohol is present at the water-cyclohexane interface and also that the free energy of transfer of an alcohol molecule from the cyclohexane continuous phase to the interface is small and negative.

(6) Based on the results of the studies reported in this chapter, we have chosen Triton X-100- alcohol (3:2 w/w), 20% w/v in cyclohexane (sometimes referred to as the 3:2 system) and Triton X-100 - alcohol (4:1 w/w), 20% w/v in cyclohexane (sometimes referred to as the 4:1 system) for further spectroscopic studies, the results of which are presented in the next chapter.

REFERENCES

1. W. Gerbacia and H.L. Rosano, J. Colloid. Interface Sci., 44, 242 (1973).
2. O.A. El Seoud, J. Chem. Soc. Perkin Trans., 2, 1497 (1976).
3. K. Shinoda and H. Kunieda, J. Colloid. Interface Sci., 42, 381 (1973).
4. S. Friberg, J. Lapezynska, and G. Gillberg, J. Colloid. Interface Sci., 56, 19 (1976).
5. P.A. Winsor, Chemical Rev., 68, 1 (1968).
6. P. Ekwall, L. Mandell, and K. Fontell, Acta Chem. Scand., 22, 373 (1968).
7. P. Ekwall, L. Mandell, and K. Fontell, Molecular Crystals and Liquid Crystals, 8, 157 (1969).
8. J.H. Schulman and D.P. Riley, J. Colloid Sci., 3, 383 (1948).
9. D.O. Shah, R.D. Walker Jr., W.C. Hsieh, N.J. Shah, S. Dwivedi, J. Nelander, R. Pepinsky, and D.W. Deamer, 'Structural Aspects of Microemulsions and Cosolubilised Systems,' SPE-5815, paper presented at Improved Oil Recovery Symposium, Tulsa, U.S.A., March, 1976.
10. See 'Rheology of Emulsions' by P. Sherman in 'Emulsion Science,' Ed. P. Sherman, Academic Press, New York, 1968.
11. J.W. Falco, R.D. Walker Jr., and D.O. Shah, AIChE Jour., 20, 510 (1974).
12. A. Einstein, Ann. Physik, 34, 591 (1911).
13. R. Roscoe, Brit. J. Appl. Phys., 3, 267 (1952).
14. R.H. Sweeney, and R.D. Geckler, J. Appl. Phys., 25, 1135 (1954).
15. F.L. Saunders, J. Colloid Scie., 16, 13 (1961).

16. See also, 'Rheology of Emulsions,' Ed. P. Sherman, Pergamon Press, London (1963).

17. See, 'Scattering of Light and Other Electromagnetic Radiation,' M. Kerker, Academic Press, New York, 1969.

18. See 'Light Scattering', by G. Oster in (p. 85) 'Physical Methods of Chemistry', Vol. 1, part IIIA, Eds. Weissberger and Rossiter, Wiley Interscience, New York, 1972.

19. J.H. Schulman, W. Stoecknius and L.M. Prince, J.Phys. Chem. 83, 1677 (1959).

20. W. Stoecknius, J.H. Schulman and L.M. Prince, Koll. Zeil, 169, 170 (1960).

21. J.E. Bowcott and J.H. Schulman, Zeitschrift. fur Electrochemie, Ber. Bunsenges. Physik. Chem., 59, 283 (1955).

CHAPTER III*

SPECTROSCOPIC STUDIES ON TRITON X-100 - ALCOHOL MICROEMULSIONS

*Two papers based on the work presented in this chapter are to be published as:

(1) C. Kumar and D. Balasubramanian, J. Colloid. Interface Sci. (in Press), 1980.

(2) C. Kumar and D. Balasubramanian, J. Phys. Chem., (Manuscript submitted).

In this chapter we present the results of a number of different spectral studies directed towards an elucidation of the molecular organisation of the Triton X-100 - hexanol-cyclohexane-water microemulsion system.

As pointed out in the Introduction, while simple reverse micelle systems have been extensively studied by spectroscopic techniques, the only microemulsion system studied in some detail so far is the potassium oleate-hexanol system. Since the bulk physical properties of the Triton X-100 - alcohol system are very different from those of simple reverse micelles, we have utilised the various spectral techniques developed in connection with studies on the latter systems to compare the features of the two at the microstructural level. We believe that many of these techniques are being utilised on a mixed surfactant microemulsion system for the first time and that many different such microemulsion systems will have to be studied by these techniques before generalisations can be made about the molecular organisation of microemulsions, especially in view of the microemulsion - swollen micellar solution controversy as documented earlier.

EXPERIMENTAL

The purification of the solvent cyclohexane, Triton X-100, alcohols and water were as mentioned earlier in Chapter II. The dyes used were purified by repeated recrystallisation and salts used were of the highest purity available. 8-Anilinonaphthalene

sulfonic acid, a product of Sigma, was converted to the magnesium salt and recrystallised from water after charcoal treatment. The ESR spin probe used was synthesized according to Rozantsev and Neiman (1).

UV-Vis absorption spectra were run on a modified Toshniwal RL02 UV-Vis spectrometer or a Cary-17D spectrometer. Near IR spectra were also run on a Cary-17D spectromter. λ_{max} values presented were reproducible to within $\pm$0.5 nm. Fluorescence spectra were run in a simple double monochromator fluorescence photometer assembled in the laboratory. Quantum yields were calculated according to standard methods (2). The λ_{max} values obtained in the fluorescence spectra were accurate to $\pm$1 nm and quantum yields to $\pm$0.01. Fluorescence polarisation and depolarisation of scattered light were measured in a suitably modified Brice Phoenix DM-2000 light scattering photometer.

The pK_a of dyes were measured in aqueous solution by dissolving the dye in solutions of successively increasing pH and recording the visible spectra. From the spectra in very low and very high pH solutions, the molar extinction coefficient and λ_{max} of the dyes in the protonated and deprotonated forms were estimated. pK_a was calculated from the ratio of protonated and deprotonated forms at intermediate pH values according to

$$pK_a = pH + \log \frac{\text{deprotonated dye}}{\text{protonated dye}}$$

and the average pK_a calculated. For estimation of pK_a of dyes

in the microemulsions, a similar procedure was adopted. Here the pH of the water pool was taken to be the pH of the aqueous solution of dye that was added to the organic components of the system to form the microemulsion. pH, in these cases, was adjusted by conc. HCl or NaOH.

All NMR spectra were run on the Varian XL-100 NMR spectrometer at the Regional Sophisticated Instrumentation Centre, IIT-Madras, while the relaxation time measurements were performed on a JEOL FX-100 FT-NMR spectrometer at the University of Hyderabad. The chemical shifts are expressed in δ units with TMS as internal reference. The T_1 measurements were carried out with D_2O as external lock by the inversion recovery method under the control of the FX-100 Autostacking program.

All measurements reported were carried out at 25 $\pm$ 1°C.

While many of the experiments were carried out with the three alcohols, pentanol, hexanol and octanol playing the role of the cosurfactant in the Triton X-100 - alcohol system, the results are presented essentially for the Triton X-100 - hexanol system. Qualitatively similar behaviour was obtained with the other two alcohols also and the overall interpretation of the results with hexanol as cosurfactant holds.

RESULTS AND DISCUSSION

A. Near IR Studies

One of the overtones of the basic vibrational frequencies of the bulk, normally hydrogen bonded liquid water occurs at

1.93 μ in the near infrared region (3). Since this band is clearly separated from the many other vibrational modes of the water molecule, this band offers a convenient handle for the study of the state of water in reverse micelles and other similar systems (4).

The near infrared spectrum of Triton X-100 : hexanol (4:1, w/w) 20% w/v in cyclohexane is presented in Fig. 3.1 for low amounts of added water. The spectrum shows an important feature in the form of a shoulder at 1.89 μ at the very low water concentration of 0.2% (v/v) apart from the dominant 1.93 μ band. This shoulder weakens and vanishes into the trailing portion of the 1.93 μ band at higher water concentrations. The 1.93 μ band can be identified as the same as the one seen in bulk water, while the band at 1.89 μ is not seen in normal bulk water. In light of this, the results of Fig. 3.1, can be interpreted as follows: water which is present in the water pools within the microemulsions in this system should be closer to bulk water while water that is distributed in the cyclohexane phase should be somewhat dissimilar. Hence, we can assign the 1.93 μ band to the water in pools and the 1.89 μ shoulder to water distributed in the bulk cyclohexane phase. The relative intensities of the 1.93 μ band and 1.89 μ shoulder, judged from expanded plots, should correspond to the ratio of water in the water pools to water in the cyclohexane phase. From this it is estimated that not more than 0.02% (v/v) of water enters the bulk cyclohexane phase at any water concentration in the microemulsion system.

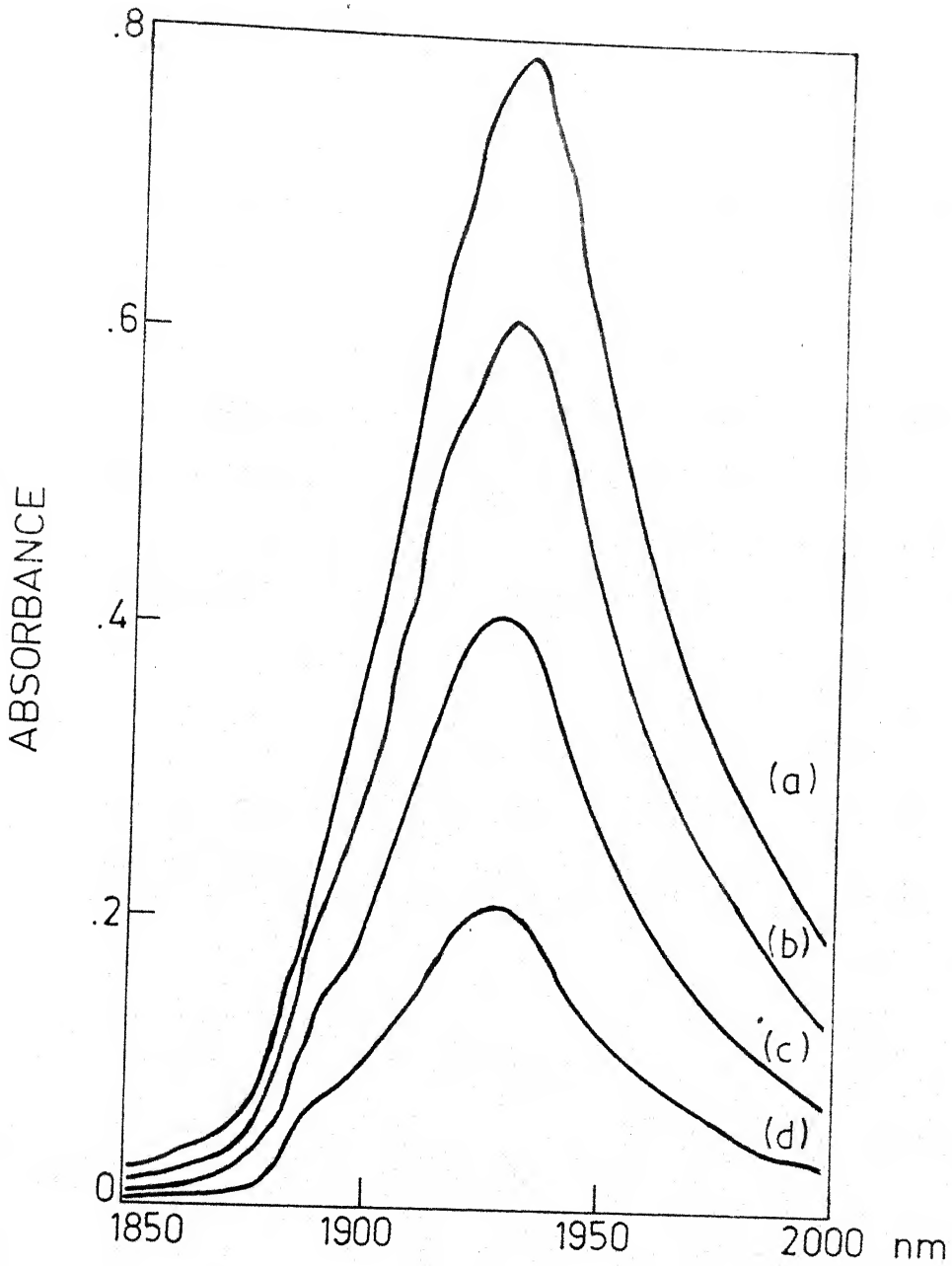


Fig. 3.1 Near IR spectra of 4:1 system at different water concentrations.

(a) —— 0.2% v/v
(b) —— 0.4% v/v
(c) —— 0.6% v/v
(d) —— 0.8% v/v

This means that at water concentrations of 1% and above the fraction of water that is distributed in the cyclohexane phase can be neglected and all the water treated as being present in the water pools. This is an important result, useful for the interpretation of other spectral studies.

These low values for the concentration of water in the bulk phase are similar to the values obtained for dodecylammoniumpropionate reverse micelles in benzene (4) and much lower than the 2.8% v/v obtained for the potassium oleate-hexanol - hexadecane system (5). These differences are probably due to the presence of different concentrations of surfactant and co-surfactant in the bulk phase.

The Triton X-100 : hexanol (3:2) system also showed similar results on near IR investigation.

B. $CoCl_2$ Absorption Studies

The divalent cobalt cation shows spectral characteristics which are very sensitive to its degree of coordination (6). While the octahedrally coordinated $\left[Co(H_2O)_6\right]^{2+}$ absorbs weakly around 520 nm in the visible, tetrahedrally coordinated cobalt shows a much more intense, red shifted absorption band around 670 nm. Hence, if Co^{2+} is introduced into a water pool, depending on its degree of hydration, its spectral characteristics will change. This property has been utilised by Wells to probe lecithin (7) reverse micelles and we have utilised the same for studying the state of water in the Triton X-100 - hexanol system.

Figure 3.2 shows the absorption spectrum of $CoCl_2$ included in the Triton X-100 - hexanol (4:1) system at a low water concentration, while Fig. 3.3 shows the molar extinction coefficient at 665 nm of cobalt in the 4:1 and 3:2 systems as a function of water concentration.

It is evident from Fig. 3.2 that at such low water concentrations, a sizable part of Co^{+2} in the system is present in the form of a tetrahedrally coordinated species, i.e., they lose at least two molecules of water from their normal octahedral sphere of hydration. Given that the water to cobalt ratio is at least 50:1 at these concentrations, there can be two reasons for the deprival of water: (1) removal of water to the cyclohexane phase, (2) the binding, competitively, of water by the surfactant head groups. As the NIR results have been used to show that the amount of water present in the cyclohexane phase is very small, surfactant head group binding to the available water must be the major reason. Hence any decrease in the A_{665} of cobalt ion is indicative of the presence of free water and as can be seen from Fig. 3.3, the amount of free water slowly increases with the total water content of the system so that at about 2% v/v water concentration in the system, all Co^{2+} is present in the octahedrally hydrated form.

From this we can conclude that water present within the Triton X-100 - hexanol microemulsion water pools is partly bound to the surfactant head groups, with the fraction of bound water decreasing from an initial high value on further additions of

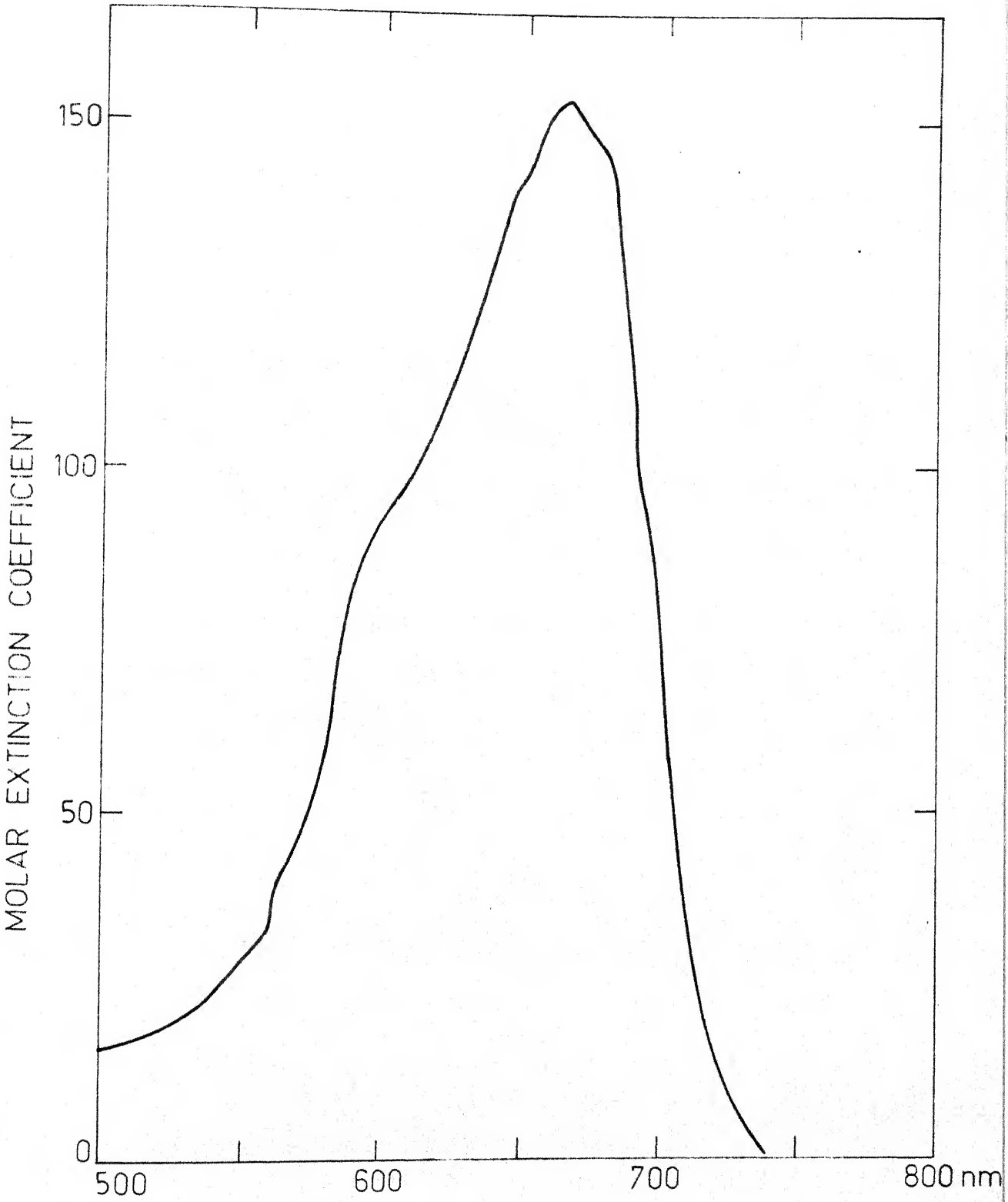


Fig. 3.2 Absorption spectrum of $CoCl_2$ in 4:1 system at 0.5% v/v H_2O added. ($CoCl_2$ concentration = 2mM overall)

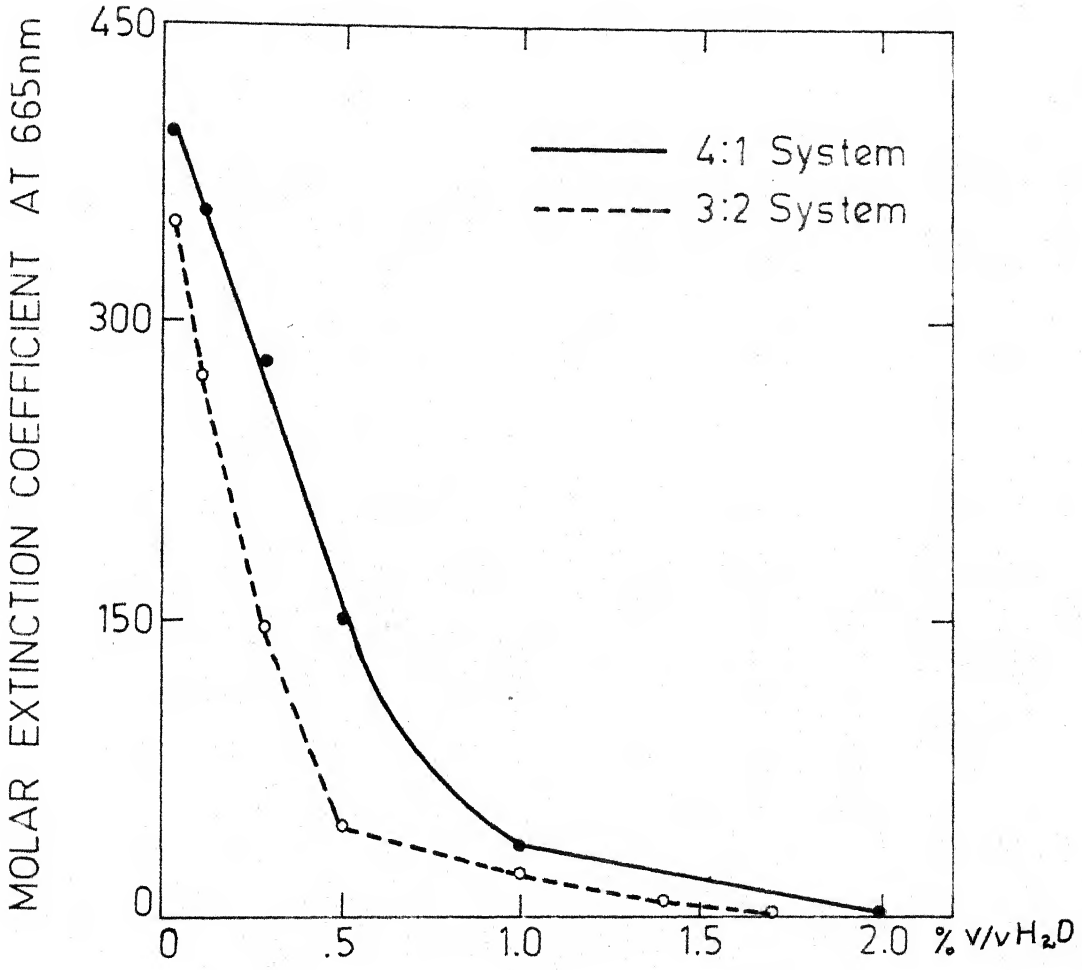


Fig. 3.3 Variation of Molar Extinction Coefficient at 665nm of $CoCl_2$ as a function of water concentration in the microemulsion systems.

water. These results are formally similar to those obtained by Wells on lecithin reverse micelles, and indicate that the initial water (upto 2%) solubilized in the Triton X-100 - hexanol microemulsions is bound to the surfactant and qualitatively different from bulk liquid water (7). It is also evident from Fig. 3.3 that the fraction of bound water is higher in the 4:1 system than the 3:2 system at any given water concentration, consistent with the higher concentration of ethylene oxide residues in the 4:1 system.

C. Acidity of Water Pools

Through a study of the absorption spectra of dyes incorporated into the water pools, it has been shown before that Igepol CO-530 reverse micelles containing small amounts of solubilised water show a basic environment to the solubilised dyes, while water solubilised in dodecylammonium propionate reverse micelles is similar to bulk water in its acidity characteristics (8).

We have tested the Triton X-100 - hexanol 4:1 and 3:2 systems for their acidity characteristics by following the absorption spectra of dyes solubilised into the microemulsion as aqueous solutions at different pH values. Thymol blue, methyl orange and malachite green, so tested, showed pK_a values in the Triton X-100 - hexanol systems within ± 0.2 pH units of their bulk water pK values of 1.65, 3.4 and 2.3 respectively. This was true of a range of different total water concentrations

from 0.5 to 11% v/v. This shows that in its acidity characteristics, water solubilised into Triton X-100 - hexanol microemulsions is not significantly different from bulk water.

On the other hand, p-nitrophenol was found to have a pK_a of above 11.0 at all water concentrations tested in these systems. This result is akin to the behaviour of p-nitrophenol in the Aerosol OT-heptane water reverse micellar system (9). But unlike the Aerosol OT system, where imidazole shifted the pK_a of a part of nitrophenol to its normal value of 7.14, imidazole had no effect on pK_a of p-nitrophenol in the present system. This would seem to suggest that the apparent pK_a shift of p-nitrophenol is probably due to its partitioning into the hydrocarbon phase in presence of the surfactants in preference to the aqueous phase.

D. Polarity Probe Studies

The environmental polarity of the solubilized water inside reverse micelles has been monitored, utilising the solvent sensitive bands of pyridine 1-oxide (10), 1-ethyl-4-carbomethoxypyridinium iodide (11), Vitamin B_{12} (12), hemin (13) etc. It has generally been found that polarity of water is very low at low water concentrations and increases to higher values at higher concentrations in these reverse micelle systems (8). Since these organic probes are large and also likely to be oriented at the interface, it is possible that they do not report the unperturbed solvent polarity of the entire water-

pool. A small, ionic, inorganic probe can be expected to dissolve in the complete water pool and report the average polarity of the entire water pool .

To this end, we have utilised the nitrate ion as a polarity probe. The nitrate ion has an n-π^* band at around 300 nm which is quite sensitive to the solvent used. The solvent sensitivity of this band seems to arise from the differences in H-bonding ability of the ground and excited states of the oxygen electrons and hence the solvent shift is a measure of the hydrogen bonding ability of the solvent (14). Figure 3.4 shows the variation in λ_{max} of the n-π^* band of nitrate ion in 3:2 and 4:1 systems as a function of water concentration. (λ_{max} of NO_3^- ion, for comparison, is 309.0 nm in $CHCl_3$ and 301.5 nm in H_2O). At low (circa 1% v/v) water concentrations, the nitrate n-π^* band appears around 306.5 nm, indicating a much lower strength of interaction between the pool water and the ion, the strength of interaction being more similar to alcohol-nitrate interaction than to simple water-nitrate interaction. As the water concentration increases, the nitrate-water interaction increases in strength, blue shifting the band to its normal value of around 301.5 nm seen in bulk water. Since at all times, the H_2O to NO_3^- ratio is at least 50 to 1, it is clear that the weakened solvent ion interaction is indicative of a lower effective polarity of the water pool, in comparison with bulk water.

E. Fluorescence Probe Studies

The use of fluorescent probes for the study of supramolecular and molecular organisation is very well established.

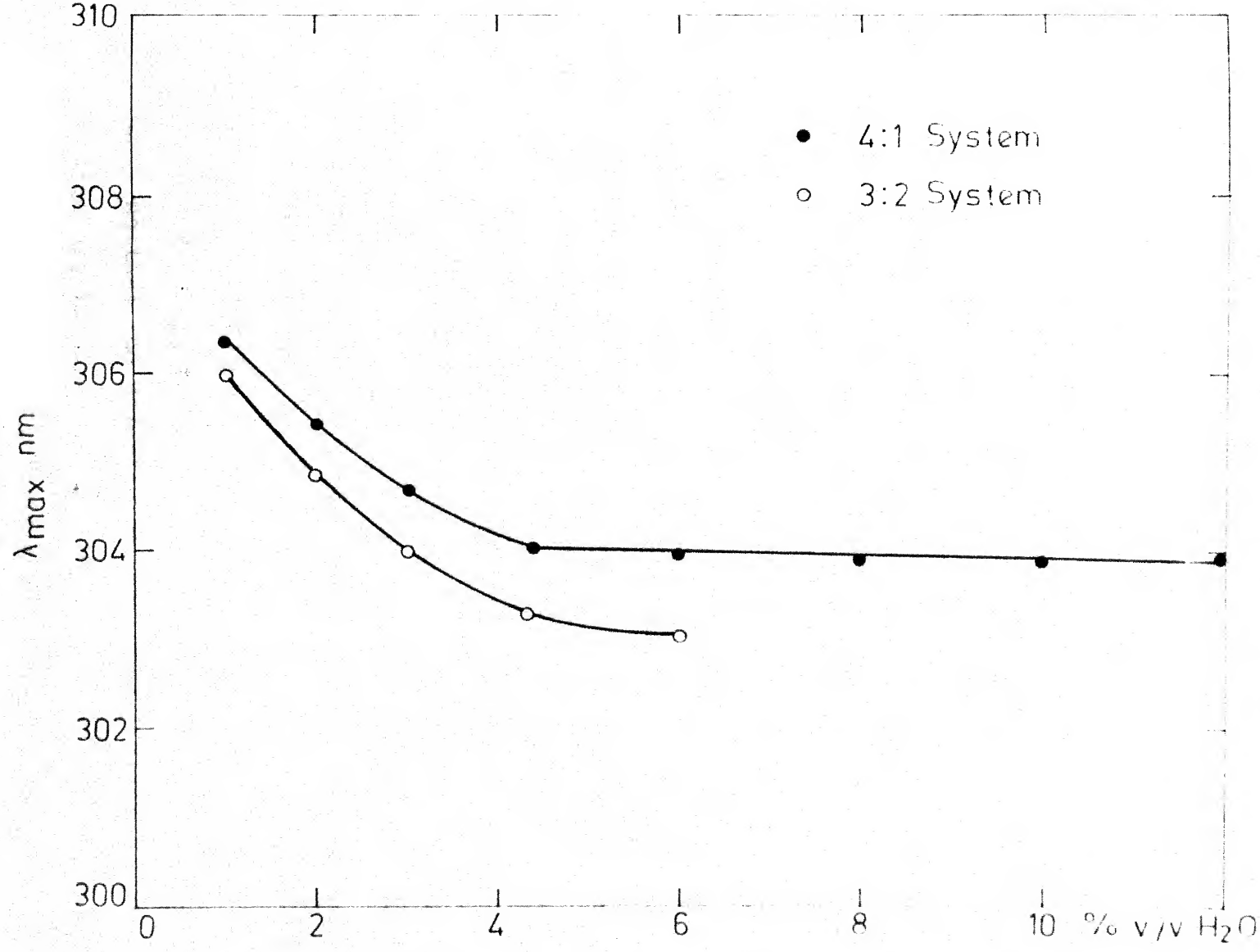


Fig. 3.4 Variation of λ_{max} of $n \rightarrow \pi^*$ band of NO_3^- as a function of added water concentration in the microemulsion system.

8-Anilinonaphthalene sulfonic acid (ANSA) is one of the extensively used fluorescence probes. The quantum yield of fluorescence and emission maxima of ANSA have been found to be well correlated with Kosower solvent polarity index, Z (15,16). The more polar the solvent, the lower the fluorescence quantum yield and more red shifted the fluorescence emission maximum of ANSA. Such behaviour has been rationalised in terms of the availability of both an excited singlet state and an excited charge transfer state to ANSA. Besides changes in solvent polarity, a second mechanism that leads to an increase in the quantum yield and a blue shift of the emission spectrum consists of factors which prevent the solvent dipole from reorienting itself about the excited state of the ANSA molecule i.e. any factor that increases the solvent viscosity (17).

Figure 3.5 shows the variation in the fluorescence emission maximum of ANSA dissolved in the 3:2 and 4:1 systems with increasing water concentration. The λ_{max} values are shown only upto about 7% v/v water concentration, since the extreme broadening of the emission band makes the fixing of λ_{max} values inaccurate above this composition. However, in both cases it can be seen that the λ_{max} red shifts upon addition of water, thereby suggesting that the polarity of the water pools in which the ANSA molecules are localised increases with increasing concentration of water. The initially large shifts suggest that at lower concentrations lower than 3% v/v, the polarity of the water pool as a whole is significantly lower than that of bulk water, a result

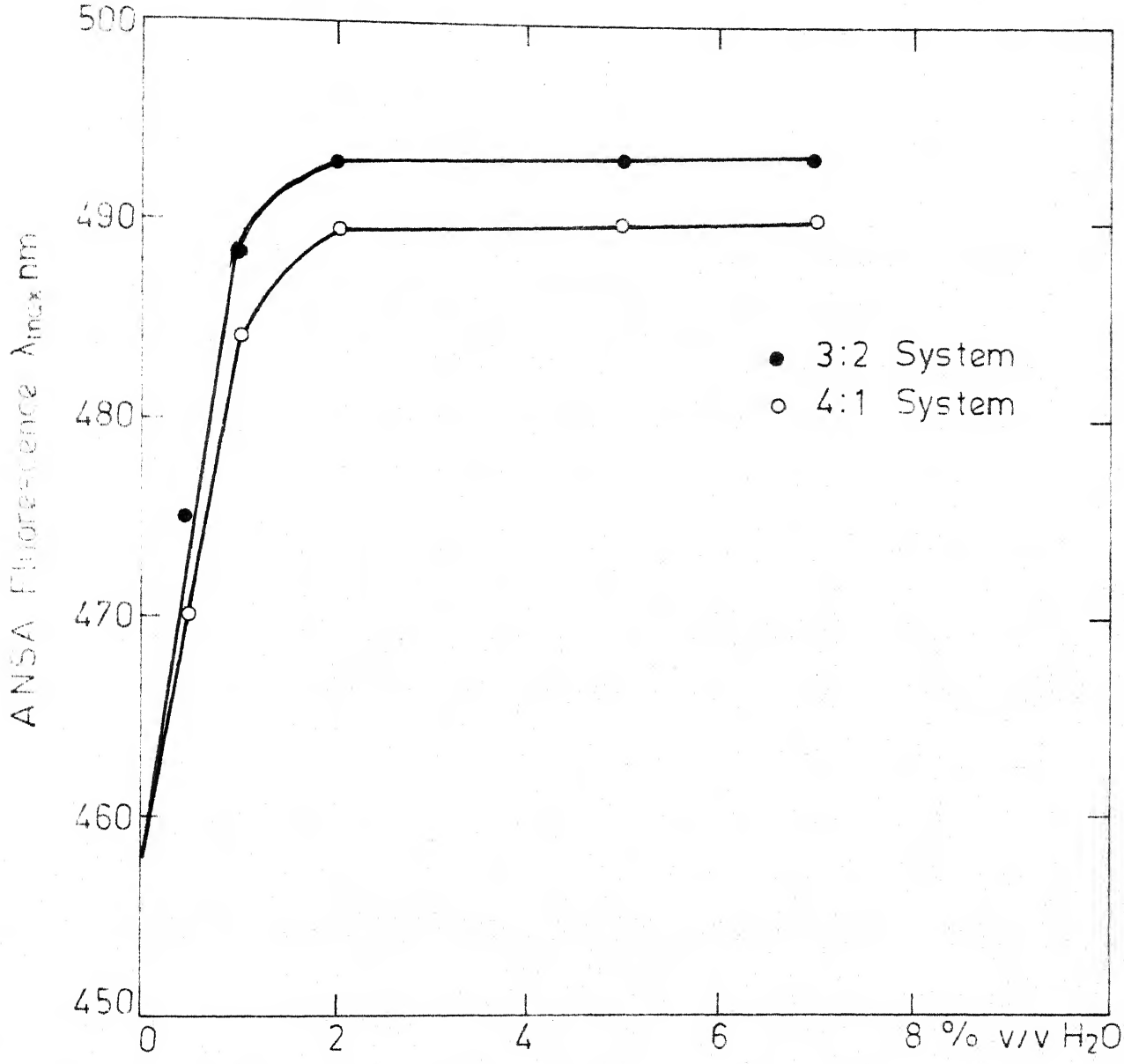


Fig. 3.5 Variation in the Fluorescence Emission maximum of ANSA as a function of added water concentration in the micro emulsion system.

in consonance with the nitrate ion studies described before.

Figure 3.6 shows the variation in fluorescence quantum yield of ANSA in 3:2 and 4:1 systems as a function of water concentration. It can be seen from Fig. 3.6 that the quantum yield of ANSA falls from a value of about 0.40 (corresponding in Z values to about 90% dioxane) to about 0.10 (corresponding to 60% dioxane) as the water concentration increases from 0 to 4% v/v. From this it would seem that at lower than 4% v/v water concentrations, the polarity of the water pool environment is significantly lower than that of bulk water. The quantum yield of ANSA in the 3:2 system decreases somewhat faster than in the 4:1 system, presumably because of the higher concentration of the ethylene oxide residues in the latter. Even at 9% v/v concentration of water, the quantum yield of ANSA is about 0.04, much higher than the normal quantum yield of ANSA in water of about 0.003. This would seem to suggest that the water pool environment that is monitored by the ANSA molecule is an intimate mixture of head group ethylene oxide residues and water partly bound to the ethylene oxide residues. This would lead to a lower effective polarity for the water pool environment, a situation somewhat similar to a highly concentrated solution of polyethylene oxide in water. Thus the fluorescence emission maximum and quantum yield values report trends similar to the nitrate probe with respect to the polarity of the water pools in the microemulsion systems.

However, in the 4:1 system, beyond 10% water concentration, the quantum yield of the fluorescence probe increase steadily.

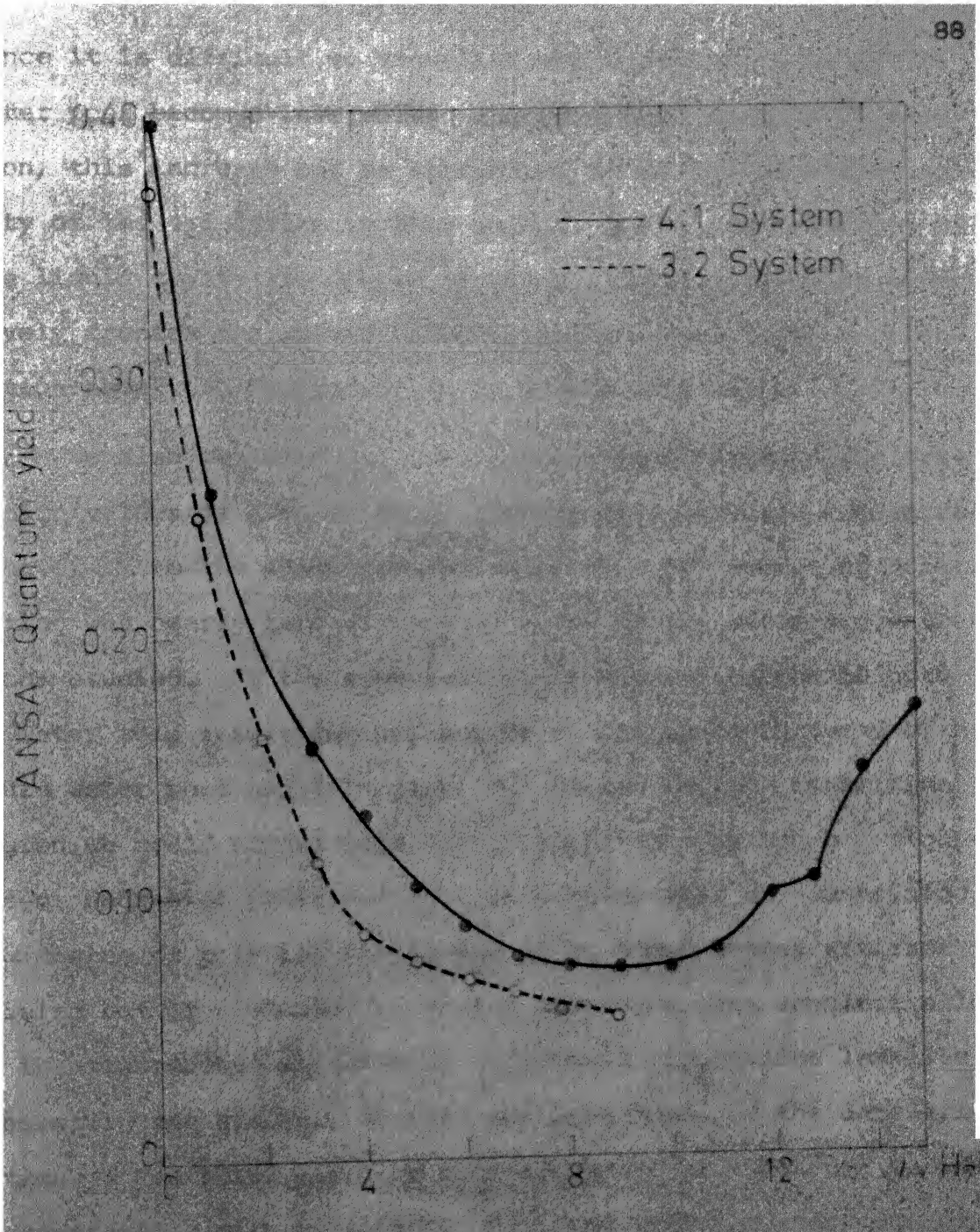


Fig 3.6 Variation of quantum yield of ANSA with amount of added water.

Since it is difficult to visualise any mechanism by which the water pool becomes less polar with increasing water concentration, this increase can be attributed to the higher microviscosity of the 4:1 system in this region, due to the formation of the liquid crystalline phase as discussed earlier, which effectively freezes the solvent dipoles and prevents their reorientation about the excited state ANSA molecules (17).

We also measured the steady state fluorescence depolarisation values of ANSA in these systems between water concentration of 1% to the phase separation point. The degree of polarisation was nearly zero (less than 0.05) at all water concentrations studied. In the spherical microemulsion region of upto 9% water this result can perhaps be rationalised in terms of a fluid water pool and interface, but in the liquid crystalline region we could expect high microviscosities and hence a significant degree of polarisation. We believe that the surprisingly low degree of polarisation is due to an experimental artifact arising out of multiple scattering and consequent depolarisation of the fluorescent emission by the liquid crystalline lamellae present in the system. To test this, we measured the depolarisation of scattered light by the 4:1 system at different water concentrations with vertically polarised incident radiation of wavelength 436 nm. It was found that light polarised through 90° is almost completely polarised upto 8% water concentration, and then the degree of polarisation falls steeply at the phase transition to the liquid crystalline phase, where it is

completely depolarised. This observation makes such an explanation of the steady state fluorescence polarisation results a tenable one.

Wong and coworkers (18) have studied Aerosol OT reverse micelles by ANSA fluorescence intensity and depolarisation of fluorescence. They found that the polarity of the water pool slowly increases and the rigidity of the water pool slowly decreases with increasing water concentration. It would seem that the Triton X-100 - hexanol microemulsions, at low water concentrations are much more fluid than the Aerosol OT reverse micelles.

F. Magnetic Resonance Studies

In order to develop an understanding of the molecular organisation of the surfactant and water molecules in our microemulsion system, we have carried out Nuclear Magnetic Resonance (NMR) and Electron Spin Resonance (ESR) studies on these systems.

Figure 3.7 shows the variation in the NMR chemical shifts of the -OH and $-CH_2CH_2O-$ protons of the 3:2 system as a function of added water concentration. As the water concentration increases from 0 to 1% v/v, the chemical shift of the ethylene oxide residues changes from about 3.88 δ to 3.93 δ and then, more slowly to around 4.00 δ as the water concentration increases to 9% v/v. This change in the chemical shift can be interpreted as follows: Initially, in the absence of water, the Triton X-100 molecules are present as a true solution, in contact with the

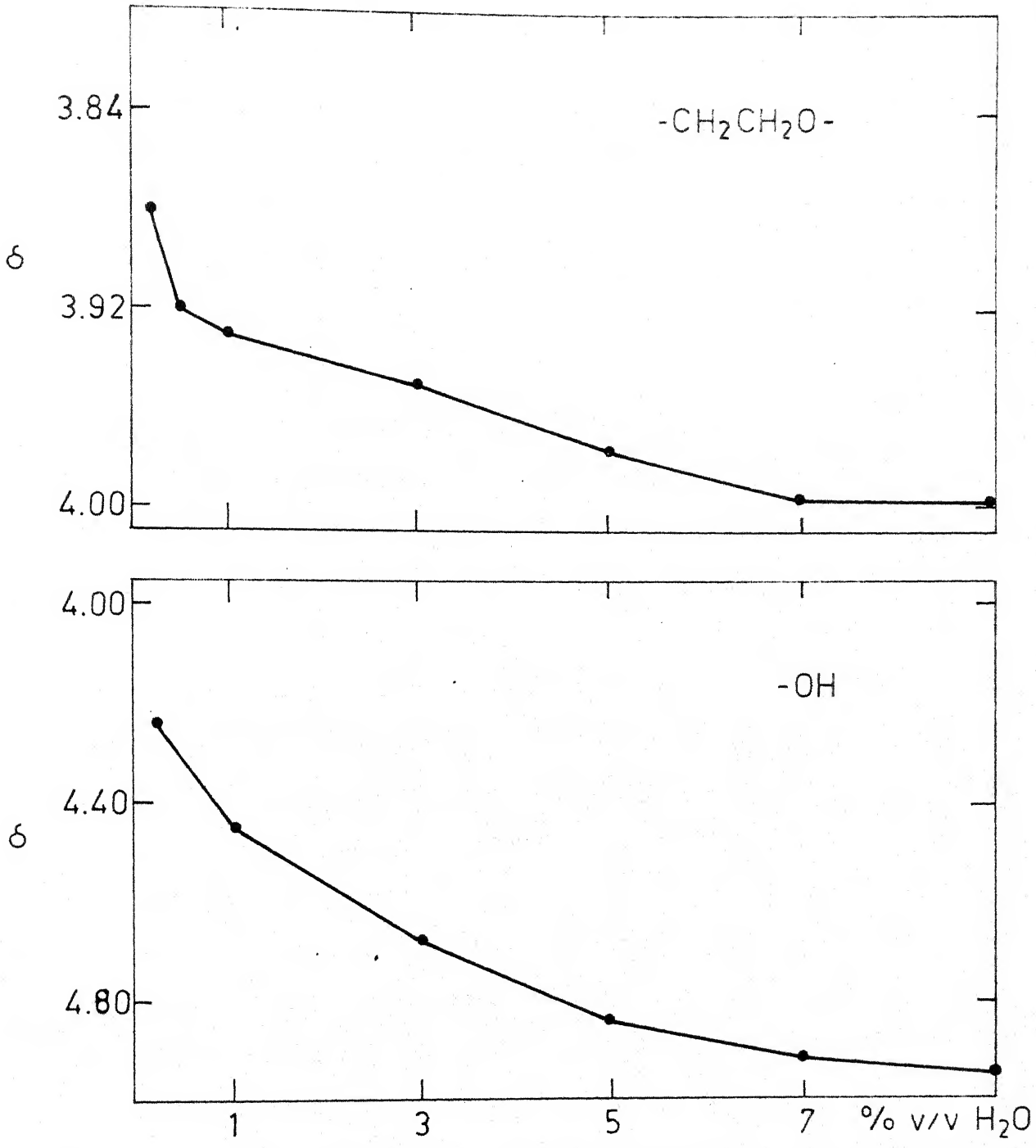


Fig. 3.7 Variation in NMR chemical shift with amount of added water : Protons in the 3:2 system.

cyclohexane solvent. On the addition of water, hexanol and Triton X-100 molecules come together to form microemulsions and the ethylene oxide headgroup is slowly removed from contact with cyclohexane and moved into the more polar aqueous environment, leading to solvent shifts in the ethylene oxide residue resonance. These results are similar to those obtained with Tween 80 reverse micelles in xylene by Gentile *et al.* (19).

The interpretation of the changes in the chemical shift of the -OH protons is complicated by the presence of the -OH protons on hexanol and the terminal -OH of Triton X-100 other than the water protons. The presence of a single signal for all these protons suggests that there is fast proton exchange between -OH of hexanol, -OH of Triton X-100 and water, as well as fast exchange between the hexanol at the interface and that dissolved in the continuous phase, cyclohexane. The chemical shift of the -OH protons will be affected by the following factors: (a) The ratio of the alcohol and Triton X-100 protons to water protons; (b) Changes in the H-bonding state and consequently the chemical shift of the alcohol proton and (c) Changes in the chemical environment of the water molecule i.e., the ratio of water bound to the surfactant *versus* free, bulk water. As there are significant changes in the position of the -OH signal even above a water concentration of 1% v/v, where water protons predominate, we can say that the first factor is not the most important one. Similarly, as it has been shown through titration experiments detailed in the previous chapter that only

a small fraction of hexanol molecules are present in the inter face factor-b can not be the predominant one. But it will mak a contribution to the overall downfield shift, as we can expec the alcohol-OH in the interface to be downshifted as the H-bon ing with water increases (20). Hence, we conclude that most o the downfield shift in the OH signal is due to changes in the chemical environment of the water molecule. This can be ratio alised as follows: in the low water concentration region, a sizable fraction of water will be bound by ethylene oxide residues. As the water concentration increases free water pools are formed, with an increase in the average level of H-bonding of water due to water-water H-bonds, leading to the downshift in the -OH resonance. These two factors, together, account for the observed shift of the -OH signal from its initial high value of 4.2 δ to the normal bulk water value of around 5.0 δ as the concentration of added water increases from 0.25 to 9% v/v.

Figure 3.8 shows the variation of the chemical shift values of the -OH and $-CH_2CH_2O-$ signals in the 4:1 system as a function of added water concentration. In the initial, 0 - 9% v/ water concentration region, the behaviour of the 4:1 system is similar to the 3:2 system and the same explanations are valid. One interesting observation is that at the phase transition to the liquid crystalline system at above 10% v/v water concentration, the water and ethylene oxide resonances do not show any significant changes. This leads one to the conclusion that the

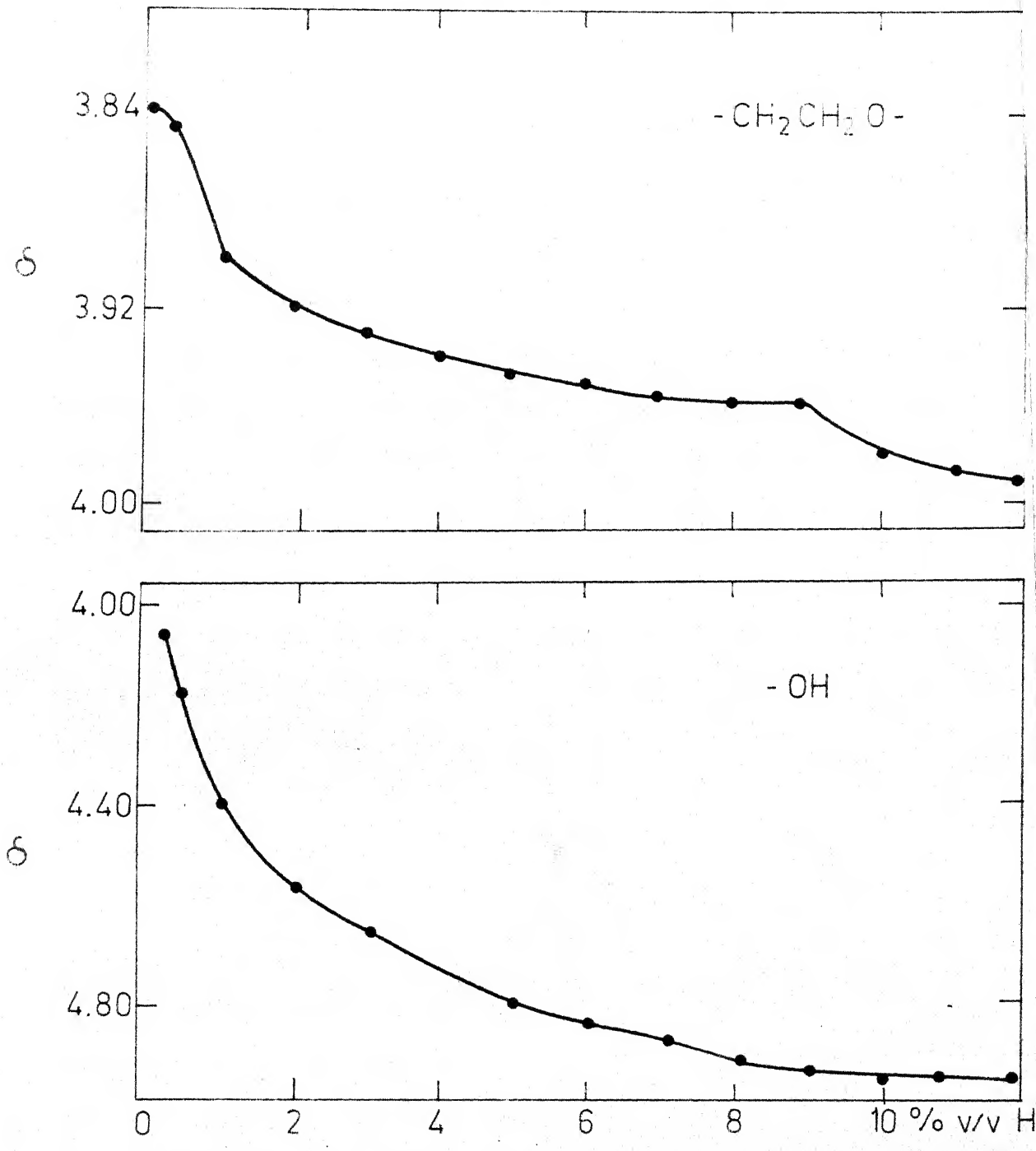


Fig. 3.8 Variation in NMR chemical shift with amount of added water: Protons in the 4:1 system.

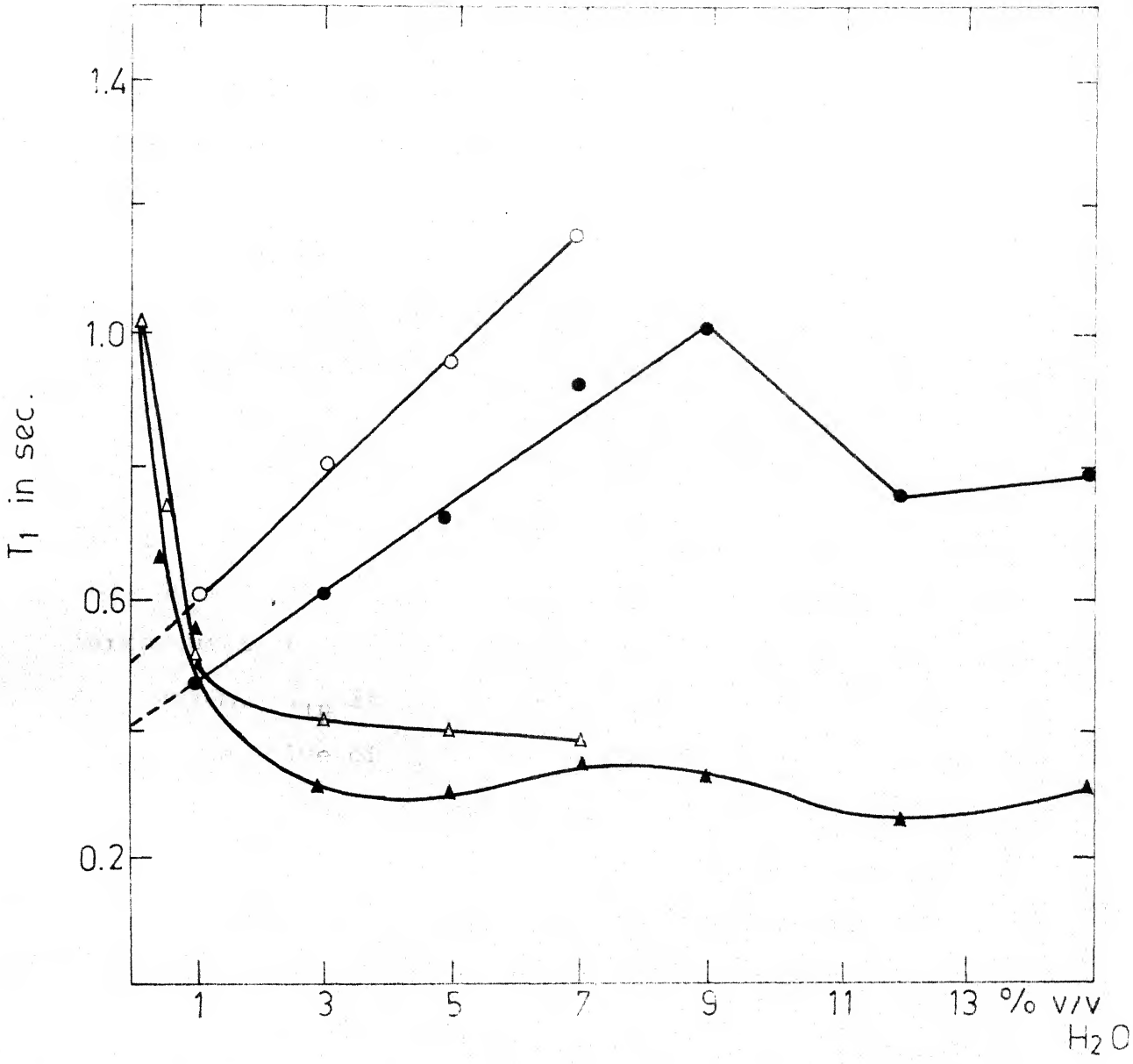


Fig. 3.9 Variation of T_1 with amount of added water

-OH protons

o 3:2 system

● 4:1 system

$-CH_2CH_2O-$ protons

△ 3:2 system

▲ 4:1 system

the core of the microemulsion water pool. Let us call this fraction f_B. These two fractions will have two different spin lattice relaxation times T_{1A} and T_{1B}. Under conditions of fast exchange (21):

$$\frac{1}{T_{1(OBS)}} = \frac{f_A}{T_{1A}} + \frac{f_B}{T_{1B}} \qquad \ldots (1)$$

If we assume that T_{1A} and T_{1B} are approximately constant in the linear T_1 range of water concentrations, we can calculate f_A and f_B if T_{1A} and T_{1B} are known. We have assumed that T_{1A} is equal to the intercept of the T_1 versus water concentration curve extrapolated to zero water concentration. This value turns out to be 0.5 sec. for the 3:2 system and 0.45 sec. for the 4:1 system. T_{1B} is set equal to the T_1 of bulk water, 3.6 sec. (22). The value of f_A thus calculated, after adjustment for the hydroxylic protons of Triton X-100 and hexanol, give the amount of water immobilised, or, in other words, bound to the Triton X-100 : ethylene oxide residues. These values are shown, as a function of water concentration in Fig. 3.10.

Figure 3.10 shows that the hydration of the ethylene oxide residues increases slowly for both the 3:2 and the 4:1 system with water concentration in the microemulsion region. The degree of hydration, expressed as a ratio of moles of water bound/mole of ethylene oxide reaches a maximum of 0.55, where it levels out (corresponding range in f_A varies from 0.82 at 1% water concentration to about 0.3 at 7% water concentration). This value

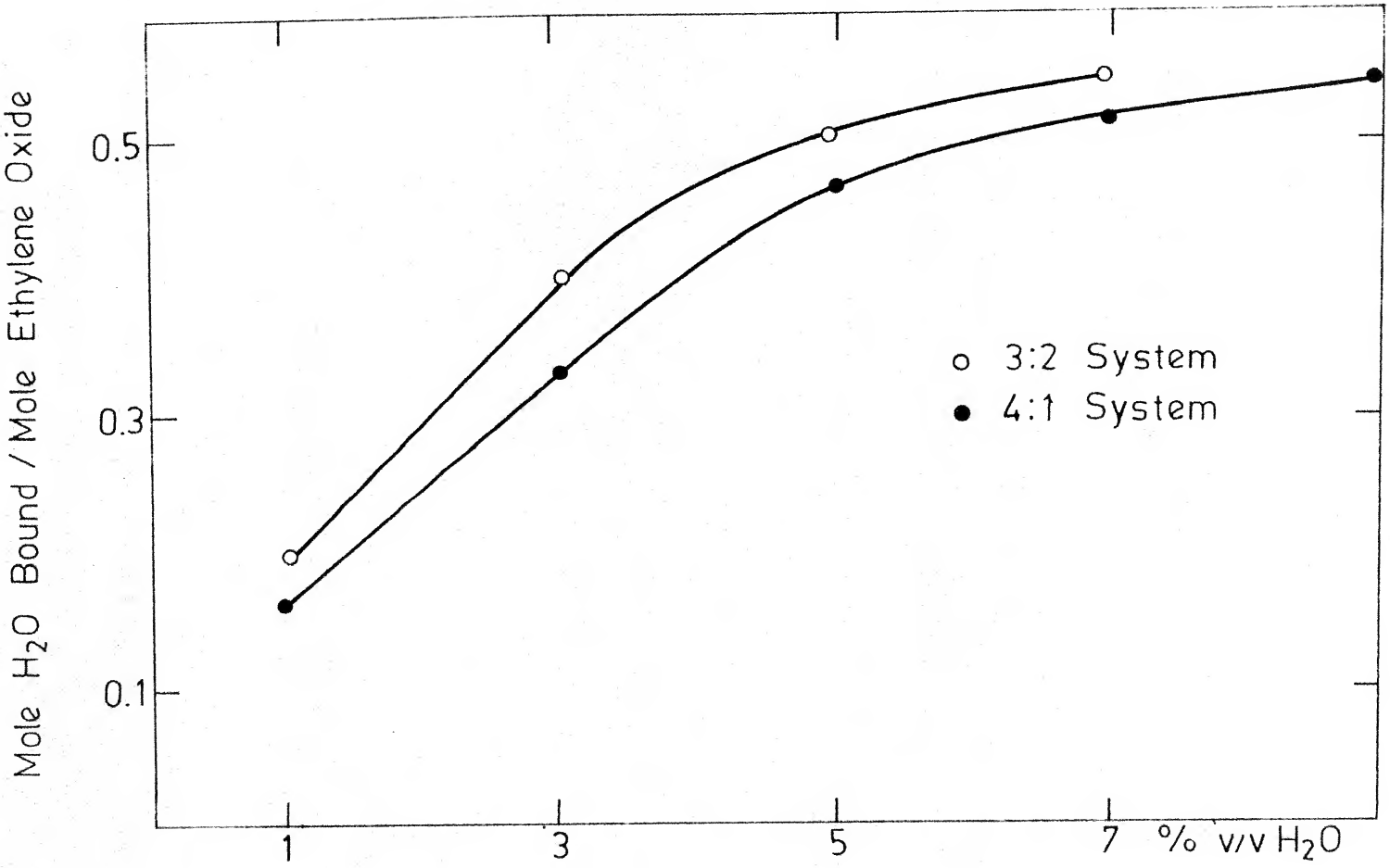


Fig. 3.10 Hydration of ethylene oxide residues as a function of amount of added water.

is significantly different from the estimates of hydration of aqueous micelles of polyoxyethylene surfactants through ESR (23) of 1 mole H_2O/mole ethylene oxide and the much higher values obtained through light scattering (24,25). The present estimate is somewhat closer to the estimate of hydration of a similar surfactant in reverse micelles in cyclohexane, the value being about 0.66, arrived at through a study of water quenching of the surfactant fluorescence (8).

Hansen has utilised a similar method to analyse the NMR data on D_2O solubilised in potassium oleate-hexanol microemulsions (26). He showed that a monomolecular film of D_2O at the interface is immobilised through binding to surfactant in that system. Wells (7) found that six moles of water per mole of lipid were immobilised at the interface in lecithin reverse micelles in ether.

Complementary to the above discussion, the T_1 values of the hydroxylic protons can also be interpreted in terms of microviscosity values. Such a discussion of the T_1 values is given below:

For any small molecule, the relaxation time T_1 can be considered to be the result of a combination of intermolecular and intramolecular relaxation effects, each of which can be theoretically evaluated. The relevant equations, as developed by Bloembergen, Purcell and Pound (27) are:

$$\frac{1}{T_{1\ \text{intra}}} = \frac{3}{10} \frac{\gamma^4 \hbar^2}{b^6} \left[\frac{\tau_c}{1+\omega^2 \tau_c^2} + \frac{4\tau_c}{1+4\omega^2 \tau_c^2} \right] \quad \ldots (2)$$

$$\frac{1}{T_{1\ inter}} = \frac{9\pi^2\gamma^4\eta\hbar^2 N_o}{5\ kT} \qquad \ldots (3)$$

$$\frac{1}{T_1} = \frac{1}{T_{1\ inter}} + \frac{1}{T_{1\ intra}} \qquad \ldots (4)$$

for the water molecule where $T_{1\ intra}$ and $T_{1\ inter}$ are the intramolecular and intermolecular parts of the dipolar mode of relaxation, γ is the gyromagnetic ratio, b is the proton-proton distance in the water molecule, τ_c is the rotational correlation time, ω, the resonance frequency of the water molecule, η, the microviscosity and N_o, the volume concentration of spins. Combining the value of τ_c so obtained with the Debye equation,

$$\tau_c = 4\pi\eta a^3/3\ kT \qquad \ldots (5)$$

where a is the radius of the molecule approximated to a sphere, we can estimate η, the microviscosity of the water molecules whose T_1 is known. Such a calculation has been carried out for the hydroxyl T_1 values in two ways: (a) under the simplifying assumption that the intramolecular relaxation mode alone is dominant, from Eqns. 2 & 5, and (b) under the assumption that both intra- and intermolecular relaxation mechanisms are operative, from Eqns. 2, 3, 4 and 5. The results of these calculations are listed in Table 3.1.

Table 3.1 shows that the microviscosity of water in these systems is initially high and falls with further additions

TABLE 3.1

System and water added in % (v/v)	τ_c according to Eqn. (2), 10^{-10} S	η from τ_c according to Eqn. (2) (CP)	η according to Eqn. (4) (CP)
3:2 System			
1	0.29	7.4	4.2
3	0.23	6.7	3.3
5	0.19	5.9	2.8
7	0.16	4.0	2.3
4:1 System			
1	0.39	9.7	5.5
3	0.31	7.7	4.4
5	0.26	6.6	3.8
7	0.19	4.8	2.7
9	0.18	4.4	2.5
12	0.23	5.9	3.4
15	0.22	5.5	3.1

Constants used: $b = 1.58 \times 10^{-8}$ cm; $a = 1.5 \times 10^{-8}$ cm;
$N_o = 6.75 \times 10^{22}$.

of water. In the 4:1 system, at the phase transition from the microemulsion to liquid crystalline phase, the microviscosity of the water molecules increases abruptly. Nevertheless, the estimated microviscosity of the water environment is significantly less than the bulk viscosity of the liquid crystalline phase, earlier presented in Chapter II.

It must be stressed again here that the above discussion is only a description in different words of the bound water - free water analysis presented before. Bound water has a lower mobility than free water and consequently, in this interpretation shows a higher microviscosity. Hence, the higher the fraction of bound water, higher the microviscosity estimated.

The ethylene oxide proton T_1 values, as seen from Fig. 3.9, fall considerably during the initial addition of water, then are more or less steady through the clear microemulsion region, and, in the 4:1 system, well into the liquid crystalline region. This would suggest that the fluidity of the ethylene oxide residues reduces significantly during the initial hydration and interface formation, but does not change much after that, even at the phase transition to the liquid crystalline region. The phase transition would seem to affect the fluidity of the water molecules at the microemulsion interior rather more than that of the interface.

During a past study of aqueous Triton X-100 micelles at 220 MHz, it was found that the signals from different residues in the polyoxyethylene chain could be partly resolved and the corresponding different T_1 values estimated (28). In our studies

on these microemulsions at 100 MHz, we did not achieve any such separation and the T_1 values are averages for the entire ethylene oxide chain.

The spin-spin relaxation times, T_2, are presented in Fig. 3.11 for the -OH and $-CH_2-CH_2-O-$ protons in the 3:2 and 4:1 systems as a function of water concentration. The changes in T_2 values roughly parallel the changes in T_1 values with the following major differences: (a) The magnitude of the T_2 values are of an order of magnitude lower than the T_1 values. This difference would seem to indicate the presence of other modes of relaxation in addition to the intramolecular dipole-dipole interaction. In the case of hydroxylic protons, the difference in T_1 and T_2 values may be contributed to by the exchange of protons between the alcohol and water sites. In the case of the ethylene oxide protons, the T_1-T_2 differences could be due to diffusional changes in the orientation of the surfactant at the interface, a process that has been shown to affect T_1 and T_2 values differently (29). (b) The second difference is that in the case of the 4:1 system, the T_2 values start decreasing at around 8% v/v water concentration, even though the transition from clear microemulsion to liquid crystalline phase occurs only after 11% water concentration. This suggests that some of the molecular organisational rearrangements necessary for the phase transition precede the transition itself.

The signals from the alkyl group of the surfactants could not be isolated and analysed clearly because of overlap between

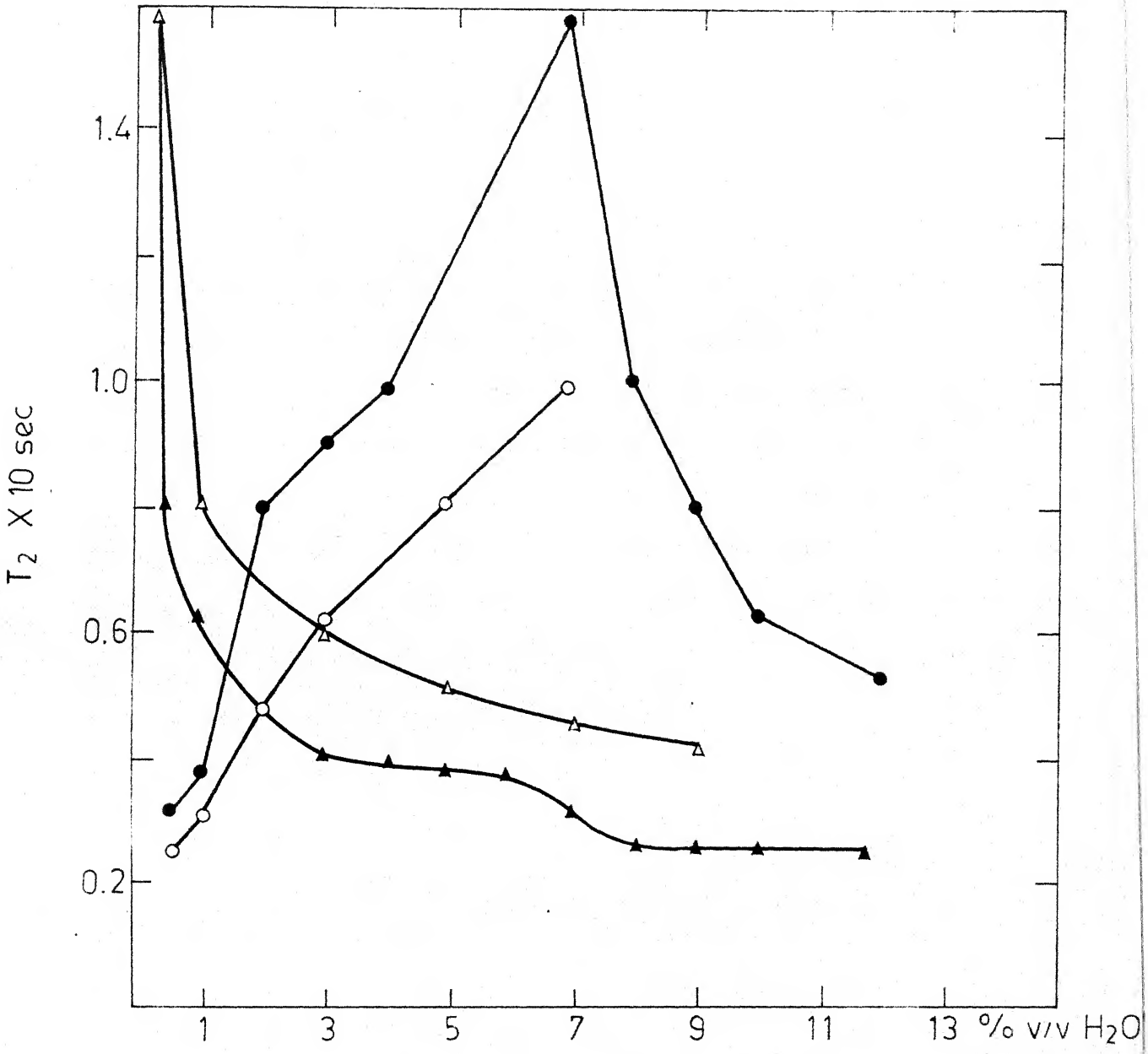


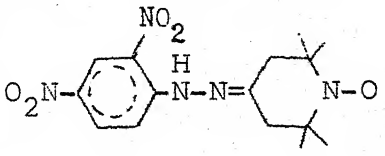
Fig. 3.11 Variation of T_2 with amount of added water.

-OH protons		-CH_2CH_2O- protons	
o	3:2 System	Δ	3:2 System
●	4:1 System	▲	4:1 System

the Triton X-100 octyl group and the hexanol signals. Also, no attempts was made to study in detail the weak but well resolved signals from the four aromatic protons of Triton X-100.

Wong et al. (30) in an NMR study of Aerosol OT reverse micelles found the spin-lattice relaxation time of the solubilised water molecules to increase with increasing water concentration. Six molecules of water were bound by each sodium ion present in the surfactant. Until the hydration of the sodium ions was complete, the rigidity of the water pool was very high. The qualitative changes occurring in the -OH T_1 values in the Triton X-100 - hexanol microemulsions are similar to the Aerosol OT system. However, the τ_c values in the Triton X-100 system are not as short as found in the Aerosol OT system at comparable water concentrations. This is probably due to the absence of ion-ion and ion-dipole attractive forces in the Triton X-100 - hexanol system.

In Fig. 3.12 is presented the variation in τ_c of the ESR spin probe used ((2,4-dinitrophenyl)hydrazone of 1,2,2',2'-tetramethylpiperidin-4-one N-oxide, Structure III.1) as a function of added water concentration in the 3:2 and 4:1 systems:



Structure III.1

The τ_c values were calculated from the ESR spectra of the above probe at very low concentration in the 3:2 and 4:1 systems

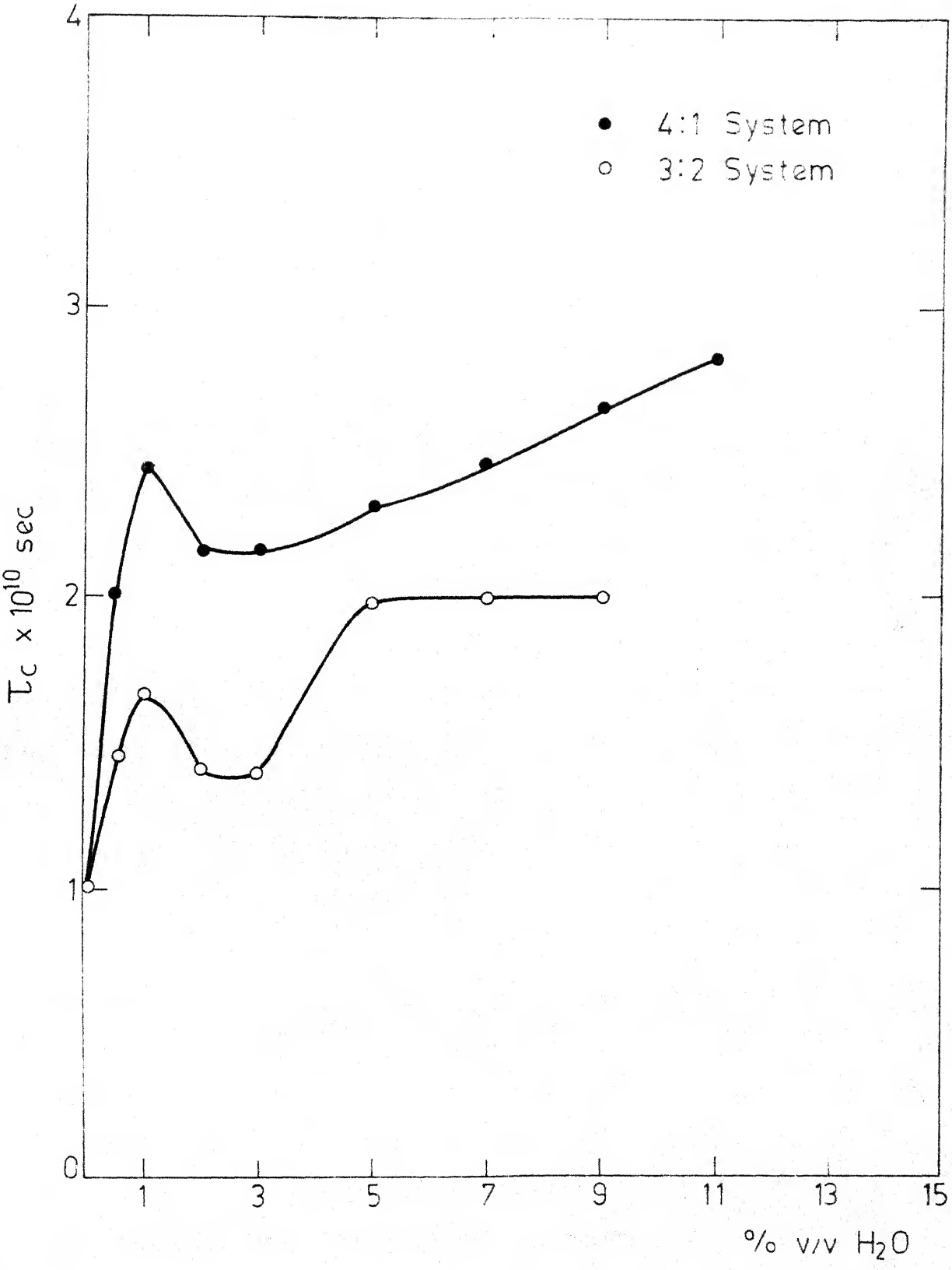


Fig. 3.12 Variation of τ_c of spin probe with amount of added wat

at different water concentrations according to Eqn. (6) (31):

$$\tau_c = A.\ \Delta H_{(m=+1)} \left[\left(\frac{I_{(m=+1)}}{I_{(m=-1)}} \right)^{1/2} - 1 \right] \qquad \ldots (6)$$

where $\Delta H_{(m=+1)}$ is the peak to peak width in gauss of the low field absorption line, $I_{(m=+1)}$ and $I_{(m=-1)}$ are the peak to peak heights for the low and high field lines respectively of the differential ESR spectrum. A is a constant whose value was set equal to 6.6×10^{-10} (31).

The other factor that needs to be considered is the position of the ESR probe within the system. That the probe is within the water pool or interface and not in the continuous phase seems likely from the way τ_c changes in a fashion not directly related to the bulk viscosity of the system. To further localise the probe within the system, we have measured the λ_{max} of the longest wavelength absorption band of the probe at different water concentrations in our systems. This band is highly sensitive to the solvent polarity, its λ_{max}, for example, being 372 nm in water and 343 nm in dodecane (23). The λ_{max} of the ESR probe in both the 3:2 and 4:1 systems varied between 357 to 360 nm with increasing water concentration. This would correspond to a slightly less polar environment than pure ethylene glycol dimethylether (23) and this suggests that the probe is positioned close to the phenyl ether end of the ethylene oxide chain. This is the only position in the entire system consistent with the estimated polarity of the environment of the spin probe.

Figure 3.1 shows that the microviscosity felt by the phenyl ether end of the ethylene oxide chain rises to a maximum, falls to an intermediate value and rises again as water concentration increases. It is roughly estimated that a change in τ_c of this probe from 1×10^{-10}S to 3×10^{-10}S corresponds to a microviscosity change from 1.6 CP to about 4.8 CP by a comparison of τ_c values of this probe in different solvents.

It must be noted that in the same range of water concentrations, the NMR data discussed earlier show that, on the average, the fluidity of the entire oxyethylene chain continuously decreases with increasing water concentration. This, when combined with the ESR data on the fluidity of the ether end of the polyoxyethylene chain, leads us to a speculative picture of the steps involved in the microemulsion formation which is presented below:

In the absence of water, the surfactant molecules are present as a true solution in cyclohexane solvent. This leads to the high mobility of the entire polyoxyethylene chain in the absence of water, as seen by NMR.

In the next stage, on addition of small amounts of water (i.e. upto 1% v/v), the surfactant and cosurfactant come together to form microemulsions in which the free ends of the ethylene oxide chains are interconnected through H-bonded water molecules. This leads to restriction on the whole polyoxyethylene chain mobility through random entanglement and both NMR and ESR show fluidity decrease.

Subsequent to this, on further addition of water, a free water pool is formed at the core of the water pool, leading to an expansion of the interfacial area. This makes the fluidity of the ether end of the ethylene oxide chain increase. Consequently τ_c of the spin probe decreases giving rise to the first maximum in the ESR microviscosity plot. Still the average fluidity of the entire ethylene oxide chain is low, because a higher number of H-bonds are forming in the free end of the ethylene oxide chain.

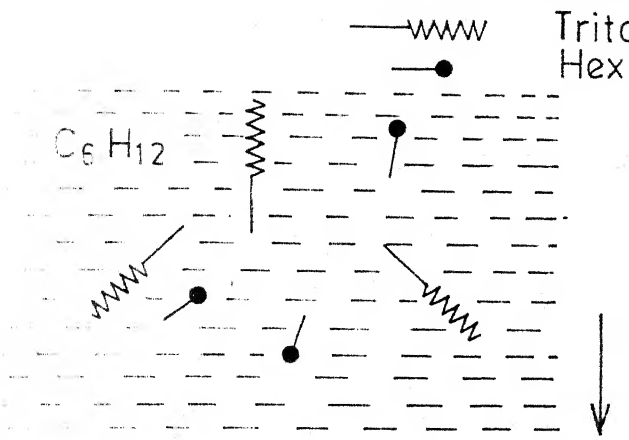
In the last stage, H-bonded water bridges are formed closer to the ether end of the ethylene oxide chain also, leading to the microviscosity increase reported by the ESR probe.

The above steps are schematically depicted in Scheme III.1.

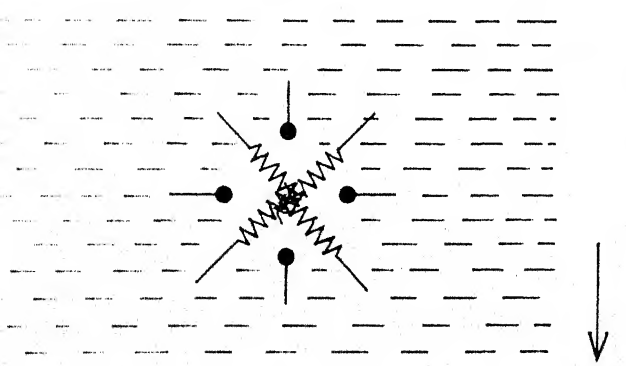
In conclusion, we have presented the results of extensive spectroscopic characterisation of the Triton X-100 - hexanol microemulsion system in this chapter. Many spectroscopic techniques developed in connection with investigations into reverse micelles have been utilised to produce similar data on our non-ionic microemulsion system.

The results show that the polarity of the water pool in Triton X-100 - microemulsions is much lower than that of bulk water. The difference in polarity between the water pool and bulk water, as monitored by ANSA fluorescence maximum, quantum yield and nitrate absorption maximum, decreases with increasing water concentration in these systems. Investigation of the extent of ionisation of dyes in microemulsion waterpools shows that

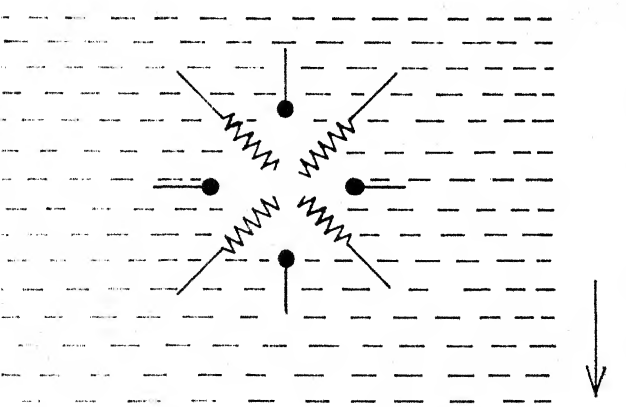
SCHEME III.1



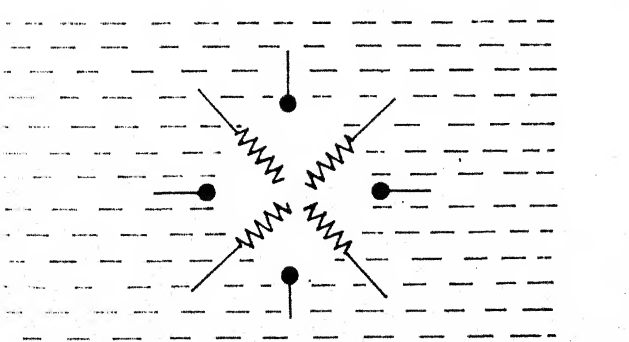
No water; free solution of surfactant. High average mobility for entire Polyoxyethylene group (long T_1, short spin probe τ_c).



Low water concentration; microemulsion with entangled polyoxyethylene group; Mobility of Polyoxyethylene deceases (lower T_1); mobility of ether end decreases (longer spin probe τ_c).



Higher water concentration; microemulsion with water pool at core. Free end of Polyoxyethylene immobilised by water through H-bonded bridges, but mobility of ether end increases (decreasing spin probe τ_c) due to interfacial area increase.



Higher water concentration, water bridges extend to ether end of Polyoxyethylene chain, reducing mobility (increasing spin probe τ_c). Water fluidity inceases due to increasing free water fraction.

the microemulsion water pools are similar to bulk water in acidity characteristics. $CoCl_2$ absorption data show that a significant fraction of water in water pools is bound to the surfactants. NMR and ESR investigations have been utilised to estimate the extent of bound water and microviscosity of the water pools as well as to develop a speculative picture of the stages involved in microemulsion formation and expansion.

The nature of the results summarised in this chapter show that even though the physical properties like size, aggregation number, total amount of water taken up etc. are very different for these microemulsion systems from the earlier studied reverse micelles, the basic molecular organisation seems to be qualitatively similar in these two cases. However, it would seem that both the water pool and surfactant sheath of the Triton X-100 - hexanol microemulsions are somewhat more fluid than the ionic reverse micellar systems which have been studied before.

REFERENCES

1. E.G. Rozantsev and M.B. Neiman, Tetrahedron, 20, 131 (1964).
2. G. Guilbault, p. 14, 'Practical Fluorescence,' Marcel Dekker, New York (1973).
3. O.D. Bonner and Y.S. Choi, J. Phys. Chem., 78, 1723 (1974).
4. M. Seno, K. Araki and S. Shiraishi, Bull. Chem. Soc. Japan, 49, 899 (1976).
5. J.W. Falco, R.D. Walker Jr., and D.O. Shah, AIChE Jour., 20, 510 (1974).
6. R.L. Carlin, p.1, 'Transition Metal Chemistry,' Vol. 1 (ed. R.L. Carlin), Marcel Dekker, New York (1965).
7. M.A. Wells, Biochemistry, 13, 4937 (1974).
8. J.H. Fendler, Acc. Chem. Research, 9, 153 (1976).
9. F.M. Menger and G. Saito, J. Amer. Chem. Soc., 100, 4376 (1978).
10. F.M. Menger, J.A. Donohue and R.F. Williams, J. Amer. Chem. Soc., 95, 286 (1973).
11. J.H. Fendler and L.J. Liu, J. Amer. Chem. Soc., 97, 999 (1975).
12. J.H. Fendler, F. Nome and H.C. Van Woert, J. Amer. Chem. Soc., 96, 6745 (1976).
13. W. Hinze and J.H. Fendler, J. Chem. Soc., Dalton Trans., 238 (1975).
14. E. Rotlevi and A. Treinin, J. Phys. Chem., 69, 2645 (1965).
15. D.C. Turner and L. Brand, Biochemistry, 7, 3381 (1968).
16. E.M. Kosower, 'An Introduction to Physical Organic Chemistry,' Wiley, New York, 1968.
17. W. Galley, p.35, 'Probes and Membrane Function' (B. Chance, C.P. Zee and J.K. Blasie, Eds.), Academic Press, New York (1971).

18. M. Wong, M. Gratzel and J.K. Thomas, J. Amer. Chem. Soc., 98, 2391 (1976).

19. F.P. Gentile, F. Ricci, F. Podo and P.E. Gna, Gazz. Chim. Ital., 106, 423 (1976).

20. J.C. Davis Jr., K.S. Pitzer and C.N.R. Rao, J. Phys. Chem., 64, 1744 (1960).

21. D.E. Woessner and J.R. Zimmermann, J. Phys. Chem., 67, 1590 (1963).

22. K. Krinicki, Physica, 32, 167 (1966).

23. H. Yoshioka, J. Colloid. Interface Sci., 63, 378 (1978).

24. R.A. Horne, J.P. Almeida, A.F. Day and N.T. Yu, J. Colloid. Interface Sci., 35, 77 (1971).

25. P. Becher and H. Arai, J. Colloid. Interface Sci., 27, 634 (1968).

26. J.R. Hansen, J. Phys. Chem., 78, 256 (1974).

27. N. Bloembergen, E.M. Purcell and R.V. Pound, Phys. Rev., 73, 679 (1948).

28. F. Podo, A. Ray and G. Nemethy, J. Amer. Chem. Soc., 95, 6164 (1973).

29. E.J. Staples and G.J.T. Tiddy, J. Chem. Soc., Farad. Trans. I, 74, 2530 (1978).

30. M. Wong, J.K. Thomas and T. Nowak, J. Amer. Chem. Soc., 99, 4730 (1977).

31. O.H. Griffith, D.W. Cornell and H.M. McConnell, J. Chem. Phys., 43, 2909 (1965).

CHAPTER IV*

STUDIES ON SOLUBILISED PROTEINS IN MICROEMULSIONS

---

*A paper based on the work described in this chapter has been communicated for publication:

C. Kumar and D. Balasubramanian, Nature, (Manuscript submitted) 1979.

Water is the basic medium in which most biochemical processes occur. Even such processes as are said to occur at membrane sites more commonly occur at the water-membrane interfaces rather than deep within the membranes, out of contact with water.

Evolutionarily, enzymes, the natural catalysts of biochemical processes, have developed so as to exhibit maximal catalytic activity in aqueous environments. Most enzymes possess flexible conformations which may undergo drastic changes depending on the environment in which they are placed. Only a very small subset of these many possible conformations possess significant catalytic properties. Normally, water is essential for the stabilisation of the active conformation of the enzymes. Changing the solvent from water to some other liquid affects the conformation of enzymes by altering the many forces responsible for maintaining their structure, namely: a) hydrophobic interactions, b) solvent-solute dipole-dipole and ion-dipole interactions and c) the effect of the changes in the solvent dielectric constant upon the dipole-dipole and ion-dipole interactions between different parts of the solute molecules.

In the light of the above, it would be rather surprising if it is found that enzymes can be solubilised into solvents that are vastly different from water with significant retention of their catalytic properties and activity. Hence study of the conformation and activity of enzymes in non-polar solvents has elicited much interest in recent years.

One approach towards the solubilisation of normally water soluble proteins into nonpolar organic media has been to utilise surfactants to extract the enzyme from an aqueous solution into an organic phase.

Das and Crane (1) found that cytochrome can be extracted from aqueous solutions into isooctane in presence of ethanol and beef heart phospholipids. This was explained in terms of the formation of a protein lipid complex which was isooctane soluble. It was suggested that the complex formation was due to electrostatic interaction of the protein with the lipid. The presence of polyvalent ions in the aqueous phase prevented complex formation and consequently the extraction of the protein into the organic phase. Such direct complex formation between protein and lipids and other surfactants has been studied more extensively in recent years in the context of protein-lipid interactions in biomembranes and it has been shown that such complexes are sometimes soluble in organic solvents. For example, Nemat-Gorgani and Dodd (2) have shown that glutamate dehydrogenase-phospholipid complexes are soluble in isooctane. Dodd and coworkers (3) have also reported that trypsin-phospholipid complexes, formed in an aqueous buffer, can be extracted into isooctane. The isooctane solution of the protein-lipid complexes was found to be clear and showed a normal trypsin spectrum. The enzyme could be re-extracted into aqueous buffer with good retention of enzyme activity. Trypsin in isooctane was more stable towards thermal denaturation than trypsin in aqueous

buffer. It was also found that many other basic enzymes could also be extracted into isooctane as complexes with phospholipids.

In a similar vein Luigi-Luisi and coworkers (4) have shown that trioctylmethylammonium chloride, a cyclohexane soluble, water insoluble surfactant, can extract tryptophane and its derivatives from aqueous solutions into cyclohexane. A study of the CD, UV and fluorescence spectra of these compounds in the cyclohexane phase suggests the presence of strong electrostatic interaction between the tryptophane indole moiety and the cationic surfactant. They later showed that α-chymotrypsin, contained in aqueous solution at pH 9.5 could also be extracted into cyclohexane by the same surfactant (5,6). UV and CD spectra of the protein in the cyclohexane phase suggested that the protein retained its native conformation.

In a later study, Luisi and coworkers (7) explored the conditions and efficiency of phase transfer to cyclohexane from water of a number of proteins and small peptides. The phase transfer efficiency was found to correlate well with the pK_a of small peptides, but not with the pI of proteins. This was interpreted to mean that small molecules are extracted into cyclohexane as electrostatic complexes with the surfactant, while for proteins the situation is more complex. These authors have suggested that the surfactant forms reverse micelles in cyclohexane in whose polar core are included the extracted protein along with some water.

The studies so far listed suffer from two serious limitations: 1) in no case was it known whether the enzyme retained its activity in the organic phase, 2) in each phase transfer, it can be envisaged that an unknown quantity of water enters the organic phase along with the protein and unless the amount of water present in the organic phase is exactly known, it is difficult to interpret any physical or chemical data that can be obtained from a study of such systems. Such a restriction can be removed by the addition of an enzyme solution to a surfactant solution in nonpolar solvent such that all the water added is solubilised into the organic phase.

Martinek and coworkers (8) have reported that chymotrypsin and peroxidase retain at least part of their catalytic activity when solubilised into octane by bis(2-ethylhexyl)-sodiumsulfosuccinate in presence of water. They utilised this system for simple synthetic purposes, but presented no quantitative results regarding enzyme activities or conformation.

Misiorowski and Wells, in an extensive study, found phospholipase A_2 to be active towards phosphatidylcholine reverse micelles in ether (9). They measured the specific activity of phospholipase A_2 in this system at precisely known water and Ca^{2+} concentrations. They found that there was an optimum water concentration where the enzyme showed maximum catalytic activity and interpreted the water concentration dependence of the enzyme activity as arising from different degrees of hydration of the phosphatidylcholine reverse micelles. The enzyme was localised

at the core of the phosphatidylcholine reverse micelles, along with the water present.

Recently Wolf and Luisi have solubilised ribonuclease in octane by the addition of aqueous solutions of the enzyme to an octane solution of bis(2-ethylhexyl)sodium sulfosuccinate (10). UV and CD spectra showed the enzyme to possess a conformation similar to that in water. Enzyme assays showed the enzyme to possess activity values that varied significantly with the pH and concentration of water in the system.

Earlier on Balasubramanian and coworkers (11) had noted that α-chymotrypsin retains part of its catalytic activity when included into microemulsions made from water, potassium oleate, hexanol and hexadecane.

It is clear from the data thus far reviewed that enzymes solubilised into organic solvents in presence of water by surfactants retain some of their enzymatic activity in many cases. This can be understood in terms of the formation of reverse micellar or microemulsion structures. Under these conditions the surfactant film at the interface may protect the enzyme from coming into direct contact with the organic solvent and thereby preserve the enzyme in its 'native' conformation.

This picture, in turn, raises many interesting questions: will all microemulsions protect all enzymes from denaturation or are there only specific combinations that will have this property? What is the relationship between the concentration of water and enzyme activity? Will microemulsions also be

effective in solubilising and protecting enzymes as reverse micelles are? As was discussed in the earlier chapter, the structure of water in water pools is not identical with that of bulk water, at least at low water concentration. Since water structure affects hydrophobic interactions strongly will this lead to changes in the conformation of the solubilised enzyme? How would the electrostatic interactions between the enzyme and the surfactant affect enzyme structure and activity?

Relevance of these studies to certain other points of interest must also be pointed out:

1. It is well established (12) that the nature of water in cells is not the same as that of 'free' water. A significant portion of water in cells, especially in the vicinity of membranes, exists as bound water. Such bound water could have significant effects on enzyme structure so that the possibility exists that enzyme conformation in these bound water regions may differ somewhat from the conformation of the same enzyme in simple bulk water solutions. As discussed in the earlier chapter, a sizeable portion of the water in the water pools in microemulsions exists as some sort of bound water. Hence enzymes within microemulsions may serve as model systems for enzymes in cellular environments.

2. Enzymes are the best catalysts known to man for many reactions. Still the utilisation of enzymes as catalysts in organic synthesis is not yet common because of a) the comparatively difficult task and high cost of purifying many enzymes; b) most enzymes are catalytically active only in aqueous

solutions, precluding their use on reactants that are soluble only in nonpolar solvents. Another problem with the requirement for aqueous environments is the fact that many equilibria, in which water is a product, are unfavourably shifted in aqueous environments. For example consider,

$$R\text{-}OH + R'COOH \longrightarrow R'COOR + H_2O$$

If water is the solvent used for the above reaction the high concentration of water in the system will shift the equilibrium far to the left and hence such ester synthesis can never be carried out conveniently in aqueous media.

All these difficulties are reduced if we can use enzymes solubilised in the core of microemulsions as catalysts. Since the water pool and the surrounding surfactant film together are much larger than the solvent molecules, they will be retained by dialysis membranes, facilitating the recovery of the enzyme used from the reaction mixture. The reactants and products will be removed with the dialysate. Since the system is largely nonpolar, substances only sparingly soluble in water can be made to react under enzymatic catalysis. Since the concentration of water in the system is adjustable down to very low values, favourable equilibria may be obtained.

In the light of these factors, we have carried out a systematic study of the activity of α-chymotrypsin solubilised into different microemulsion systems at varying, precisely controlled

water concentrations. The surfactants used were of the three charge types, cationic, anionic and nonionic, so that comparisons can be made. Further we have carried out circular dichroism studies on bovine serum albumin and chymotrypsin solubilised in the same microemulsion systems and present results related to the conformation of these proteins in the microemulsion systems used.

EXPERIMENTAL

Triton X-100, a product of Roehm Haas and Co., was purchased from the CSIR Biochemicals Unit, Delhi. Sodium dodecylsulfate, sodium laurate, cetyl trimethylammonium bromide and Brij-56(cetyl ether of polyoxyethylene-10) were from Sigma. Aerosol OT (bis(2-ethylhexyl)sodium sulfosuccinate) was from TCI.

Aerosol OT was purified by dissolving it in warm methanol, charcoal treatment, filtration and vacuum drying of the filtrate. Triton X-100 and Brij-56 were exposed to high vacuum while warm for three hours to remove any volatile impurities and used. Cyclohexane and water were purified by standard methods.

Solubilisation was achieved by adding a measured amount of the protein dissolved in a buffer (pH 7.4, 0.1 M phosphate) to a measured volume of the surfactant solution in nonpolar solvent and mixing gently for a few minutes to ensure homogeneity. All solutions were freshly prepared everyday.

CD spectra were run on a Jasco-20 CD spectrometer at the Regional Research Laboratories, Hyderabad. Mostly 0.1 mm path-length cells were used and appropriate baseline corrections were applied.

The substrate used for chymotrypsin assays was 2,4-dinitrophenyl acetate. The more common 4-nitrophenyl acetate could not be used in many of these systems, because the product p-nitrophenol was found to be completely unionised, with a consequently low absorbance, in these systems. This is somewhat similar to the observation of Menger and Saito that the pK_a of 4-nitrophenol is shifted to above 11 in water/aerosol OT/heptane (13).

The method of assay used was the simple Bender-Kezdy procedure (14). All experiments were carried out with suitable blanks. Appropriate corrections were applied to the initial rate of hydrolysis measured to correct for the spontaneous hydrolysis of the substrate. Absorbance values for 2,4-dinitrophenol, measured at 360 nm, were converted to millimoles of 2,4-dinitrophenol by the use of extinction coefficients measured under conditions identical to the enzyme runs.

Absorbance measurements were carried out on a Cary-17D or a Toshniwal RL02 spectrophotometer. All assays were run at 25 $\pm$ 1°C and CD spectra were run at room temperature.

RESULTS AND DISCUSSION

Figure 4.1 shows the variation in the activity retained by α-chymotrypsin when solubilised into Triton X-100 : hexanol

(4:1 w/w), 20% w/v in cyclohexane as a function of added water concentration. The activity retained values are expressed as percentages of the specific activity of the same enzyme towards 2,4-dinitrophenyl acetate in aqueous buffer, pH 7,4. The activity values have been calculated after applying necessary corrections to compensate for the spontaneous hydrolysis of the substrate in the system.

Figure 4.1 shows that the percentage activity retained by α-chymotrypsin in this system is nearly zero upto 1% v/v water concentration, but rises to about 50% as the water concentration increases further to 5% v/v. Later, above 7% v/v water concentration, the percentage activity values fall to about 25%.

Such variation in activity retained by the enzyme as a function of changing water concentration can be accounted for by the following reasons:

1. As was discussed earlier, the nature of water present in the solubilised 'water pools' in the system Triton X-100 - hexanol-cyclohexane is different from that of bulk water. A significant fraction of water in the water pools is present as bound water, a state quite different from free bulk water. The fraction of water present as bound water decreases as the total water concentration increases.

Hence at low water concentration in the Triton X-100 micro-emulsions, the enzyme will be placed in an aqueous environment whose nature is significantly different from that of bulk water. This may be causing such structural perturbations in the enzyme

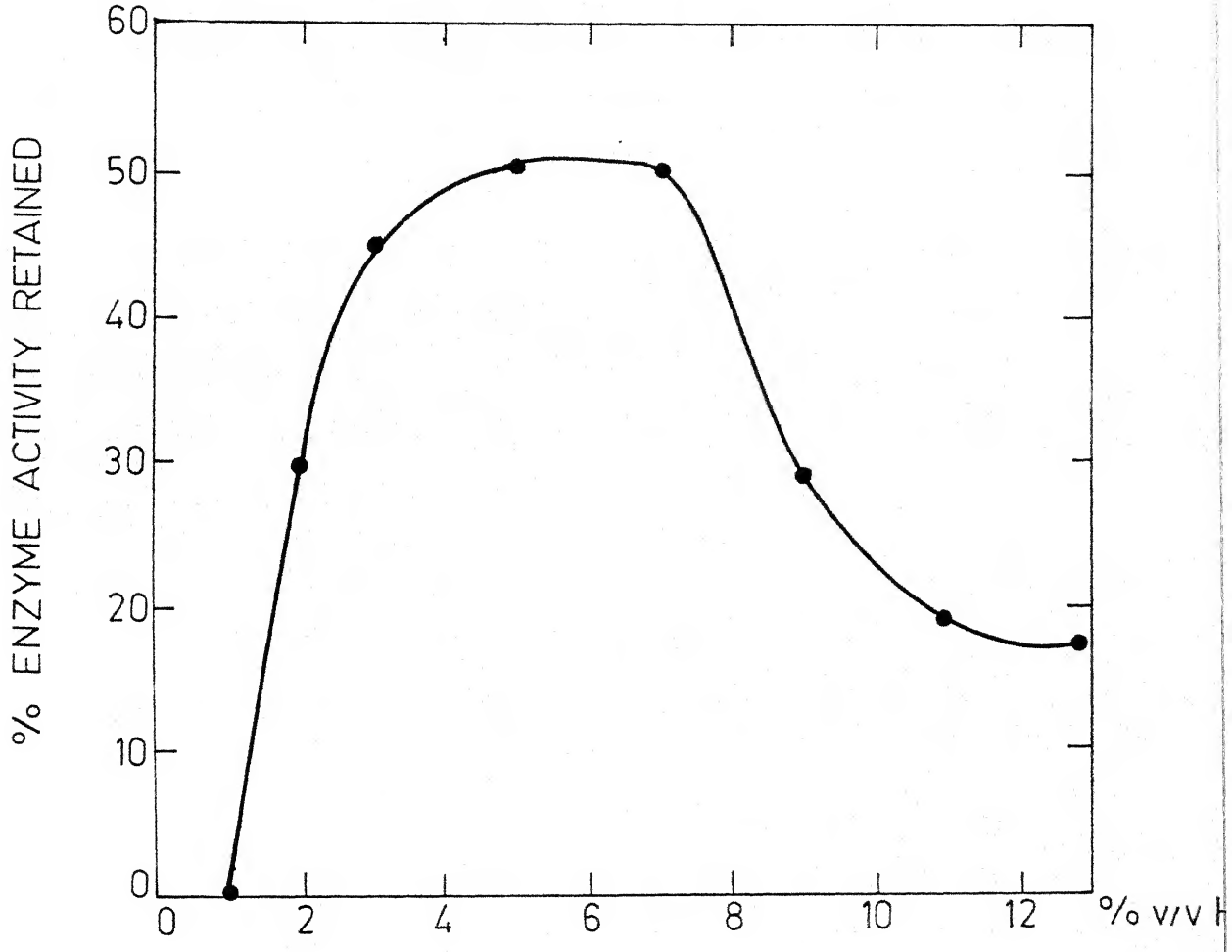


Fig. 4.1 Variation in activity of α-chymotrypsin with amount of water added in the Triton X 100 - Hexanol (4:1) system.

so as to make the enzyme lose its activity. As the total water concentration increases, the amount of free water increases and normal, active conformation of the enzyme is restored, leading to an increase in the percentage activity retained.

2. It can be envisaged that the enzyme in the water pool is distributed between the interface and aqueous core. The enzyme at the interface may be more susceptible to denaturation of sorts through contact with nonpolar solvent or surfactant residues. As the water pool increases in size, the ratio of interfacial area to the volume of the aqueous core reduces, thereby reducing the loss of enzyme activity at the interface.

3. To a limited extent, the activities measured may not be consistently representative of the catalytic ability of the solubilised enzyme since the concentration of the reactant and products in the aqueous cores of the microemulsion may not be constant through the series. Such a situation can arise if the partition coefficient of the molecules between the aqueous and organic phases changes with water concentration. However, this factor may not be very significant as the assays were run at substrate concentrations that produced maximal velocities with the enzyme present. Similarly the concentration of water is also far higher than the amount of water consumed during the reaction as well as the substrate concentration. Hence, the changes in water concentration through the series of measurements cannot explain the changes in activity retained in any direct stoichiometric way.

4. Another factor that could be of importance is the change in the viscosity of the medium or the fluidity of the interface, both of which can conceivably affect the diffusion characteristics of the substrate, products and the enzyme in the medium and this can in turn affect the measured kinetic parameters.

Of these various factors the initial increase in enzyme activity between 1% and 5% v/v water concentrations is most likely due to increasing bulk water fraction and decreasing interfacial denaturation. The fall in activity above 7% v/v water may be due to the increasing viscosity of the medium just before and following the microemulsion to liquid crystal phase transition that occurs in this system, the details of which were presented in Chapter II.

Due to the high absorbance of the substituted phenyl group in the surfactant molecule in the regions of interest, we could not investigate the CD spectral characteristics of the enzyme solubilised in this system. Nevertheless it seems likely that the native conformation of the enzyme is retained in this system at least to the extent that enzymatic activity is.

Table 4.1 presents the values of percentage activity retained by α-chymotrypsin in a number of microemulsion systems at different water concentrations. The systems were chosen so as to contain anionic, cationic and nonionic surfactants so that the effect of the charge of the surfactant on enzyme activity can also be investigated.

Table 4.1

Activities are reported at 25 ± 1°C w.r.t. standard activity of enzyme in aqueous buffer pH 7.4 towards DNPA.

System	Water v/v concn.	Enzyme (α - chymotrypsin) activity w.r.t. water*
Aerosol OT (5% in octane)	1%	0%
	3%	32%
	5%	51%
Sodium laurate (5% + hexanol 10% in C_6H_{12})	2% and above	0%
Cetyl trimethyl ammonium bromide (5% + hexanol 10% in C_6H_{12})	2% and above	0%
Sodium dodecyl-sulfate (5% + hexanol 10% in C_6H_{12})	2%	59%
	3%	85%
	5%	85%
Brij-56 (15% + hexanol 6% in C_6H_{12})	0.5%	65%
	1%	87%
	3%	86%

*Accurate to ± 10%.

Aerosol OT = di(2-ethylhexyl)sodium sulfosuccinate.

Brij-56 = cetyl ether of polyoxyethylene-10.

The results presented in Table 4.1 reveal that the enzyme α-chymotrypsin retains part of its activity in Aerosol OT reverse micelles. Catalytic ability is retained to a larger extent in sodium dodecyl sulfate and Brij-56 systems. Activity, however, is totally lost in sodium laurate and cetyl trimethylammonium bromide systems.

α-Chymotrypsin has an isoelectric point of 8.4. Hence we can expect that at the pH of the buffer forming the water pool, the enzyme will be positively charged. So, purely electrostatic considerations would suggest that the anionic surfactants Aerosol OT, sodium dodecyl sulfate and sodium laurate will bind to the enzyme electrostatically, thereby probably positioning the enzyme close to the interface. The positively charged cetyl trimethylammonium bromide will electrostatically repel the protein while Triton X-100 and Brij-56 will be neutral to the charge on the enzyme. Such electrostatic interactions were found to be of crucial importance in protein-surfactant complex formation by Dodd and coworkers (2,3).

In the case of α-chymotrypsin in microemulsions we find that of the three anionic systems used, laurate leads to total loss of enzyme activity, Aerosol OT preserves activity partly and sodium dodecyl sulfate preserves the activity of the enzyme nearly completely. Similarly, the cationic system, cetyl trimethylammonium bromide, destroys enzymatic activity completely. From these observations, it is clear that electrostatic interactions alone are not capable of determining the ability of any

microemulsion system to preserve the catalytic activity of an enzyme solubilised in it.

The nonionic Brij-56 system works best among these surfactants in preserving enzyme activity. One possible reason is that nonionic microemulsions, probably due to the absence of surfactant charge-charge interactions, are larger in size than ionic microemulsions. This would lead to a significant reduction in interfacial denaturation of the enzyme. Another point of relevance could be the bulky polyoxyethylene head group, which can act as an effective interfacial barrier, preventing the enzyme from contact with the more nonpolar part of the surfactant and the bulk solvent. Also, the percentage enzymatic activity retained normally increases with increasing water concentration in all systems which show some retention of activity initially. However, as seen with the Triton X-100 system, phase transitions may significantly affect the percentage activity retained.

Luisi and Wolf (10) have reported that ribonuclease solubilised into aerosol OT reverse micelles in octane under certain conditions showed enzymatic activity higher than that in water. In our studies with α-chymotrypsin we never found any such instance. Moreover, it was observed that the enzyme that had been solubilised in any of these microemulsion systems was irreversibly denatured on vigorous agitation. Another observation was that the activity retained by the enzyme varied from run to run slightly, to a maximum of about 10% of the average of the measured activity values.

In order to gain more direct information about the conformations adopted by proteins in microemulsion environments we have taken recourse to CD spectral investigations. Since CD spectra in 250-200 nm region are excellent indicators of protein backbone conformation, these studies should provide direct evidence regarding the conformational status of proteins in microemulsion environments.

Due to the low molar ellipticity of α-chymotrypsin and since we were restricted to very low (0.1 mm) pathlength cells due to surfactant absorption in the region of interest, we could get reliable α-chymotrypsin CD spectra only at somewhat high water concentrations. The other protein investigated, bovine serum albumin, could however, be studied at lower water concentrations as well. The CD spectra recorded were somewhat noisy, leading to uncertainties in the measured ellipticity values of about $\pm$ 10%.

Representative CD spectra of α-chymotrypsin and bovine serum albumin in different microemulsion environments as well as in simple aqueous buffer in the region 250-200 nm are presented in Figs. 4.2 and 4.3. The results are qualitatively classified and summarised in Table 4.2.

Generally three different kinds of CD spectra were obtained. The spectra which correspond to "conformation retained" classification in Table 4.2 were essentially similar to the aqueous solution spectra in position of maxima, ellipticity values and other features of the spectrum. The ellipticity

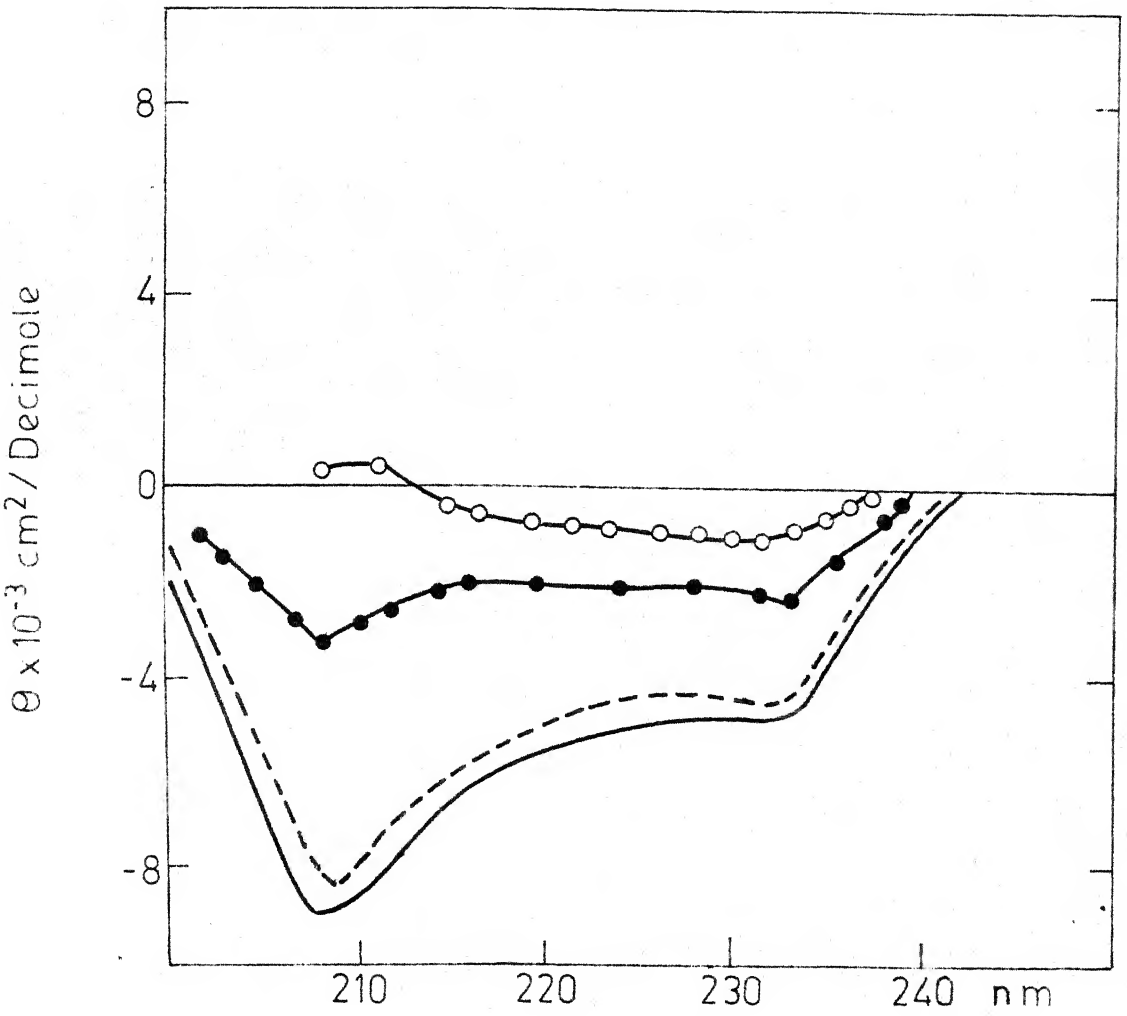


Fig. 4.2 CD spectra of α-chymotrypsin in different microemulsion and in water

——— α CT in water

------- α CT in Brij 56 system: 5% v/v water

● α CT in Aerosol OT system: 5% v/v water

o α CT in Laurate system: 5% v/v water.

Table 4.2*

System	Water concn. v/v	Conformation according to CD[a]	
		Bovine Serum Albumin	α-Chymotrypsin
1. Aerosol OT	1%	Modified	
	3%	Modified	
	5%	Modified	Partly retained
2. Sodium laurate	2%	Modified	
	5%	Modified	Modified
3. Cetyl trimethyl-ammonium bromide	2%	Modified	
	5%	Modified	Modified
4. Sodium dodecyl sulfate	2%	Retained	
	5%	Retained	Retained
5. Brij-56	0.5%	Retained	
	1%	Retained	
	3%	Retained	Retained
	5%	Retained	Retained

* System compositions identical with Table 4.1.

a. Modified: all features of the water CD spectrum of protein are lost in system and a low ellipticity spectrum obtained.

Retained: CD spectrum of protein in given system is very similar in structure and magnitude to CD spectrum in water.

Partly retained: CD spectrum of protein in given system is similar in structure but lower in magnitude compared to CD spectrum in water.

values of these CD spectra matched the corresponding aqueous CD spectra within experimental error. The classification 'partly retained' refers to spectra which were similar to the aqueous spectra in general features like wavelength of maxima, but showed lower values of molar ellipticities, of the order of 50% of the aqueous solution values. The 'modified' classification belongs to featureless CD spectra of very low (circa 1×10^3 cm^2/decimole) values of ellipticity. These spectra bear no resemblance to the corresponding aqueous spectra.

We can qualitatively interpret the three classes as follows: In those systems where the protein is essentially solubilised in its native, aqueous solution conformation, we get spectra that belong to the 'conformation retained' group. The 'modified' spectra arise from completely denatured protein conformations. The 'partly retained' kind of spectra may arise from either a conformation intermediate between the native, aqueous state and the denatured state or, more probably, from a mixed population of protein molecules, one set of molecules denatured and the other in an essentially native conformation. The latter possibility is what would be expected if, in the microemulsions, some of the protein molecules are denatured at the interface while the rest of the protein molecules, at the core of the water pools, maintain their native structure intact.

When we compare the CD spectral data obtained on α-chymotrypsin in these systems with the enzyme assays, we see that the CD data support the assay values as far as the conformation of

the enzyme is concerned. The CD spectra show that the native conformation of α-chymotrypsin is retained in sodium dodecylsulfate and Brij-56 systems, is partly retained in the Aerosol OT system and is lost in laurate and cetyl trimethylammonium bromide systems. This is the same pattern as obtained in the case of enzyme assays.

In the case of bovine serum albumin also a similar pattern is followed except that in the Aerosol OT system also the native conformation of the protein is lost. In this case again we see that charge considerations alone are not sufficient to predict the effect of the surfactant constituting the microemulsion on the conformation of the solubilised protein. Bovine serum albumin, with an isoelectric point of 4.8, will be negatively charged under the conditions of our experiment (buffered water pool, pH 7.4). We find that of the negatively charged surfactant systems used, Aerosol OT and laurate lead to loss of the native conformation of the albumin while sodium dodecyl sulfate protects the native conformation. Here again the nonionic Brij-56 is seen to be highly effective in protecting the conformation of the solubilised protein.

The CD and assay data together suggest that the nonionic Brij-56 microemulsion system is more effective than the ionic microemulsion systems considered in solubilising proteins with good retention of their native structures. The charged microemulsion systems are seen to behave differently with respect to different proteins and in a manner not explicable through charge considerations alone.

It must be further stressed here that both the activity estimates and CD spectral analysis are in themselves only partly capable of characterising the very complex conformations of proteins. Hence further studies by other available spectroscopic methods on these systems with a view to delineating the conformation of the proteins may show up more subtle differences between the conformations adopted by proteins in simple aqueous environments and the microemulsion environment.

Lastly, an experiment was carried out to test the utility of the microemulsion system for synthetic purposes. Martinek and coworkers (8) had earlier utilised chymotrypsin solubilised in Aerosol OT reverse micelles for a simple synthesis. We have carried out a similar experiment with the Triton X-100 microemulsion system. For enzyme solubilisation for synthetic purposes, the high retention of native protein conformation and the larger range of water concentrations possible with nonionic microemulsions should make them superior to reverse micelles. Moreover, microemulsions can be easily made stable at different temperatures and other conditions by simply altering the surfactant-cosurfactant ratio. Another advantage of using nonionic microemulsions is that at low water concentrations the water pools in nonionic microemulsions seem to be more fluid than the cores of ionic reverse micelles, as discussed in Chapter III. This higher fluidity should lead to faster kinetics for many reactions of interest While nonionic reverse micelles may share many of these properties with nonionic microemulsions, the small association number and

low water solubilisation capacity of most nonionic reverse micelles restrict their utility severely.

The synthetic reaction studied was the simple esterification of 2,4-dinitrophenol by acetic acid:

$$(NO_2)_2\text{-}C_6H_3\text{-}OH + CH_3COOH \rightarrow (NO_2)_2\text{-}C_6H_3\text{-}OOC\text{-}CH_3 + H_2O$$

To test the extent of the forward reaction in water, a 5 mg/ml solution of α-chymotrypsin in 0.1 molar acetate whose pH had been adjusted to 7.4 was prepared. To a measured volume of this solution, enough 2,4-dinitrophenol, dissolved in the same solvent was added to give a final absorbance of 1 at 360 nm. The solution was stirred gently at 25°C for 10 hours and the absorbance at 360 nm was again measured. There was no change in absorbance, showing that very little, if any, 2,4-dinitrophenol had been converted to the ester. This means that the equilibrium for the esterification reaction lies entirely to the left in aqueous solution.

Next, to a Triton X-100 - hexanol (4:1, w/w), 20% w/v solution in cyclohexane was added enough of a 5 mg/ml α-chymotrypsin solution in 0.1 molar acetate, pH 7.4 such that the final water concentration in the system was 2% v/v. This was gently mixed to ensure even solubilisation of the enzyme. To the solubilised enzyme system thus prepared was added enough of a 2,4-dinitrophenol solution in a miscible organic solvent to give a final absorbance value at 360 nm of 1. The solution was then

stirred gently for two hours and the absorbance was again measured at 360 nm. The absorbance value thus measured was about 0.25.

The fall in the absorbance in the microemulsion system can be ascribed to the formation of ester from 2,4-dinitrophenol and acetate. The equilibrium would seem to have been shifted from about 0% ester in water to about 75% ester in the microemulsion system under the experimental conditions employed. Since the enzyme has been earlier shown to be active in a similar system, we can assume that the equilibrium was attained with assistance from enzymatic catalysis.

The above experiment shows the utility of microemulsion systems in enzyme assisted synthesis. By forming microemulsions in appropriate solvents, many complex substrates whose solubility is low in water can be subjected to enzymatic transformations in these systems. Further, suitable conditions for achieving favourable equilibria can be attained by manipulating the concentration of water in the system. Product and catalyst recovery schemes based on dialysis through suitable membranes should be possible. All in all, it would seem that microemulsion solubilised enzyme systems are a potentially useful synthetic tool suitable for more extensive investigation.

REFERENCES

1. M.L. Das and F.L. Crane, Biochemistry, 3, 696 (1964).
2. M. Nemat-Gorgani and G.H. Dodd, Eur. J. Biochem., 74, 139 (1977).
3. P. Austin, G.H. Dodd, M. Davis and R.B. Leslie, Trans. Biochem. Soc., 2, 963 (1974).
4. A. Dossena, V. Rizzo, R. Marchelli, G. Casnati and P.L. Luisi, Biochim. Biophys. Acta, 446, 493 (1976).
5. P.L. Luisi, F.J. Bonner and C.H. Walsoe, p. 591, in 'Peptides' Proceedings of the American Peptide Symposium, Eds. M.Goodman and J. Meienhofer,John Wiley, New York (1977).
6. P.L. Luisi, F. Henninger, M. Joppich, A. Dossena and G. Casnati, Biochem. Biophys. Res. Comm., 74, 1384 (1977).
7. P,L. Luisi, F.J. Bonner, A. Pellergrini, P. Wiget and R. Wolf, Helvit. Chim. Acta, 62, 740 (1979).
8. K. Martinek, A.V. Levashov, N.L. Klyachko and I.V. Berenzin, Doklad. Akad. Nauk SSSR (Engl. Edit.), 236, 951 (1978).
9. R.L. Misiorowski and M.A. Wells, Biochemistry, 13, 4921 (1974).
10. R. Wolf and P.L. Luisi, Biochem. Biophys. Res. Comm., 89, 209 (1979).
11. D. Balasubramanian, S. Mani and C. Kumar, Nature, 254, 252 (1975).
12. W. Derbyshire in NMR, Vol. 5, Specialist Periodical Reports, pp. 264, The Chemical Society, London (1975).
13. F. Menger and G. Saito, J. Amer. Chem. Soc., 100, 4376 (1978).
14. M.L. Bender, F.J. Kezdy and F.C. Wedler, J. Chem. Educ., 44, 84 (1967).

CHAPTER V*

STUDIES ON PHOTOCONTROL OF ENZYME ACTIVITY IN A MODEL MEMBRANE SYSTEM

---

*A summary of the work described in this chapter has been published:

D. Balasubramanian, S. Subramani and C. Kumar, Nature, 254, 252 (1975).

Light being one of the most important environmental variables, many organisms have developed sophisticated light-sensitive organs that monitor changes in the light environment and influence the behaviour of the organism accordingly. A large number of processes in living organisms, ranging from the flowering of the plants to phototaxis in bacteria are under the control of such photosensitive organs. The higher organisms also utilise light for vision.

Considering the great importance of perception of and adaptation to changing light conditions, it is not surprising that many different photoregulated systems are found to exist in nature.

Hence, the study and elucidation of the mechanism of such photoregulated systems is important for the understanding of many processes. Considerable attention has been devoted to such studies in the past several years by many workers (1).

The general pattern that emerges from such studies is the involvement of specific light receptor molecules in photocontrol mechanisms. Such molecules are often proteins with light-sensitive prosthetic groups. Light of a particular wavelength is absorbed by and induces conformational changes in the prosthetic groups. The conformational changes in the prosthetic group are then transmitted to the protein part, in turn leading to changes in the enzyme activity, membrane permeability or other such

properties. Two important examples are the protein involved in vision, Rhodopsin, with its retinal prosthetic group and Phytochrome, the plant photophysiological control protein, with its tetrapyrrole prosthetic group.

In order to facilitate the study of the mechanism of such photocontrol systems, one approach that has been employed is the development of model systems that mimic some of the properties of the real systems.

Erlanger and coworkers have built model systems in which enzymes normally insensitive to light e.g. chymotrypsin (2) and acetylcholine esterase (3), have been changed into light-sensitive forms. This was achieved by conjugating the proteins to the azobenzene derivatives of their respective inhibitors. Azobenzene and its derivatives undergo cis-trans isomerisation easily upon irradiation with light of appropriate wavelength. Depending on the wavelength of the light used one can achieve cis to trans or trans to cis - isomerisation. The cis and trans forms of the inhibitors showed different inhibitory efficiencies towards the enzymes (2,3) and thus the activity of the enzyme became sensitive to light. By changing the conformation of the non-covalently bound prosthetic group (the inhibitor) photochemically, the activity of the enzyme was modulated, leading to a model photocontrol system. Similar photoregulation of the chemically induced depolarisation of electrogenic membranes using azobenzyl inhibitors has also been reported (4). Aldolase, a normally light-insensitive protein, has been converted into a

light-sensitive form by coupling the enzyme with diazotised p-aminobenzoate (5). More recently, it has been shown that the binding constants for many small molecules with cyclodextrin could be made light-sensitive by conjugating cyclodextrin with azobenzene dicarboxylic acid (6). Many such studies have been recently reviewed by Hug (7).

In the above examples, the photochromic prosthetic group directly interacts with the protein either by covalent bond formation or at specific preexisting receptor sites. Another possible way in which conformational changes in a photoreceptor molecule may affect the characteristics of a biological system is through the membranes. The photosensitive molecule present in a membrane may, upon isomerisation, affect the membrane characteristics such as fluidity. These changes may be transmitted through the membrane to enzymes located at another point on the membrane and thus the activity of enzymes may be photomodulated indirectly. One point of importance is that such a mechanism can lead to photomodulation of a host of enzymes simultaneously without invoking specific photoreceptors for each enzyme controlled.

In this chapter we present the results of our attempts to develop a model system in which membrane-mediated photocontrol of enzyme activity can be achieved.

Though these results are presented at the end of this thesis, the work contained in this chapter was carried out earliest by us and was the reason for our interest in microemulsions in general.

EXPERIMENTAL

The model membrane system we utilised was the liquid crystalline phase of the potassium oleate, n-hexanol, n-hexadecane and water system (8). All chemicals used were purified by distillation or recrystallisation from suitable solvents, as appropriate.

Conductivity measurements were carried out on a Philips PR-9500 conductivity meter at 1 KHz with platinum electrodes. The isomerisation of the photochromic molecules used, azobenzene and 4,4'-azobenzene dicarboxylic acid dimethyl ester, was achieved by illuminating the sample with a 250 W mercury lamp through filters. For cis to trans-isomerisation, irradiation was done through a Corning CS373 filter (400-500 nm) for eight minutes. For trans to cis-isomerisation, irradiation was done through a Corning CS760 filter (300-400 nm) for 8 minutes. Optical anisotropy in the system was monitored by viewing the system held between crossed polarisers against a strong light source.

The enzyme used, α-chymotrypsin, was obtained from Sigma and used as such. The substrate, p-nitrophenyl acetate, was purified by multiple recrystallisation. The assay method used was the simple Bender-Kezdy procedure (9).

To test for the binding of the photochromes to the enzyme directly, the enzyme was assayed in the presence and absence of the photochromes in a phosphate buffer of pH 8.0. If the photochrome binds to the enzyme directly, it is expected to result in changes in the activity of the enzyme.

The model membrane system was normally prepared by the addition of water to an appropriate mixture of potassium oleate, hexanol and hexadecane to achieve the desired water-to-oil ratio and gentle mixing to ensure homogeneity. Photochromes were added normally to the organic mixture before the addition of water.

For assays in the model membrane system, an aqueous solution of the enzyme was added to the organic mixture (instead of water) and gently mixed. To an aliquot of the liquid crystal containing the enzyme was added a measured volume of p-nitrophenyl acetate in hexanol. The hydrolysis of the substrate was followed by monitoring the absorbance of the solution at 400 nm at suitable intervals for 10 minutes on a Beckman DU UV-Vis spectrophotometer. The absorbance values were converted to μ moles of p-nitrophenol liberated using the extinction coefficients of p-nitrophenol measured under identical conditions. Enzyme activities were calculated after applying suitable corrections for the spontaneous hydrolysis of the substrate.

All experiments were carried out at 28 $\pm$ 1°C.

RESULTS AND DISCUSSION

The system potassium oleate - hexanol - hexadecane-water was chosen as a suitable model membrane because of its interesting properties. This system, as discussed in the Introduction, shows phase transitions from the water-in-oil microemulsion to lamellar liquid crystals to oil-in-water microemulsion as the

water:oil ratio increases. It has been shown that the phase transition properties of the system respond to Ca^{2+} and anaesthetics as do other lipid-based model membranes (10). Due to the similarity in molecular organisation of the lamellar liquid crystalline phase of this system to bilayers of lipid membranes, the lamellar liquid crystalline phase can be utilised as a convenient model membrane. The molecular arrangement of the oleate system in its different phases in shown is Fig. 5.1.

In this microemulsion system, one convenient method of following the molecular arrangement is through conductivity measurements on the system. The variation in the conductivity of the system with water:oil ratio is shown in Fig. 5.1. It will be seen from Fig. 5.1 that at low water concentrations the conductivity is very low. This corresponds to water-in-oil microemulsions. For the region of water:oil ratio 0.7 to 1.2, we find intermediate conductivity values. This region corresponds to the liquid crystal phase. Beyond this water:oil ratio, oil-in-water microemulsions are formed, with high conductivity values

To test whether the conformation of a small photochromic molecule in the model membrane system can affect the molecular organisation of the membrane, we carried out the following experiment. The liquid crystalline phase was formed from potassium oleate-hexanol-hexadecane and different amounts of water. The conductivity of the samples were measured at different water-to-oil ratios and the system irradiated for 10 min. intervals through a CS760 or a CS373 filter and the conductivity

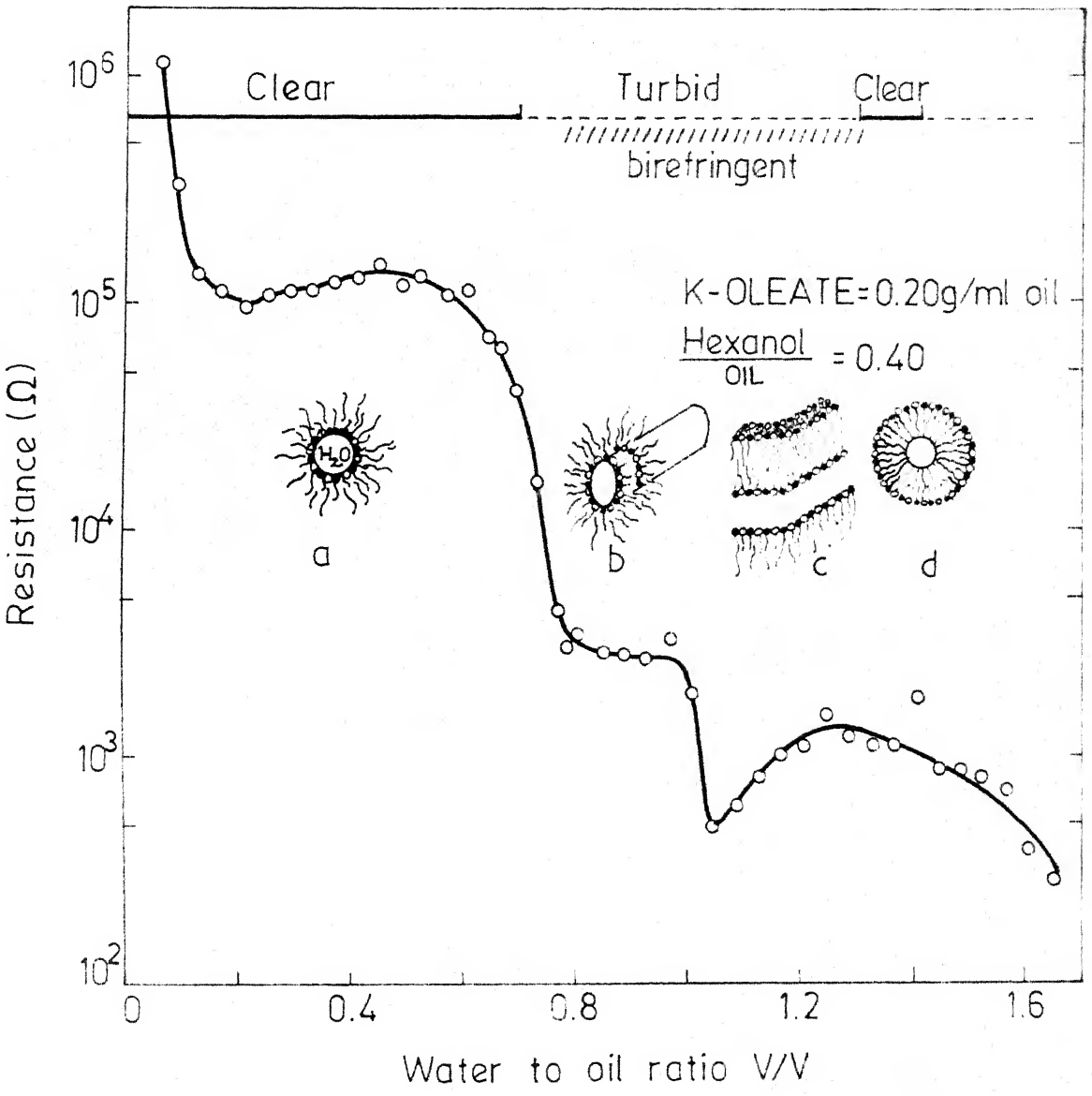


Fig. 5.1 Variation of resistance of the K-Oleate-hexanol-hexadecan water system as a function of water to oil ratio (8).

a. w/o microemulsions

b. water cylinders

c. Lamellae

d. o/w microemulsions

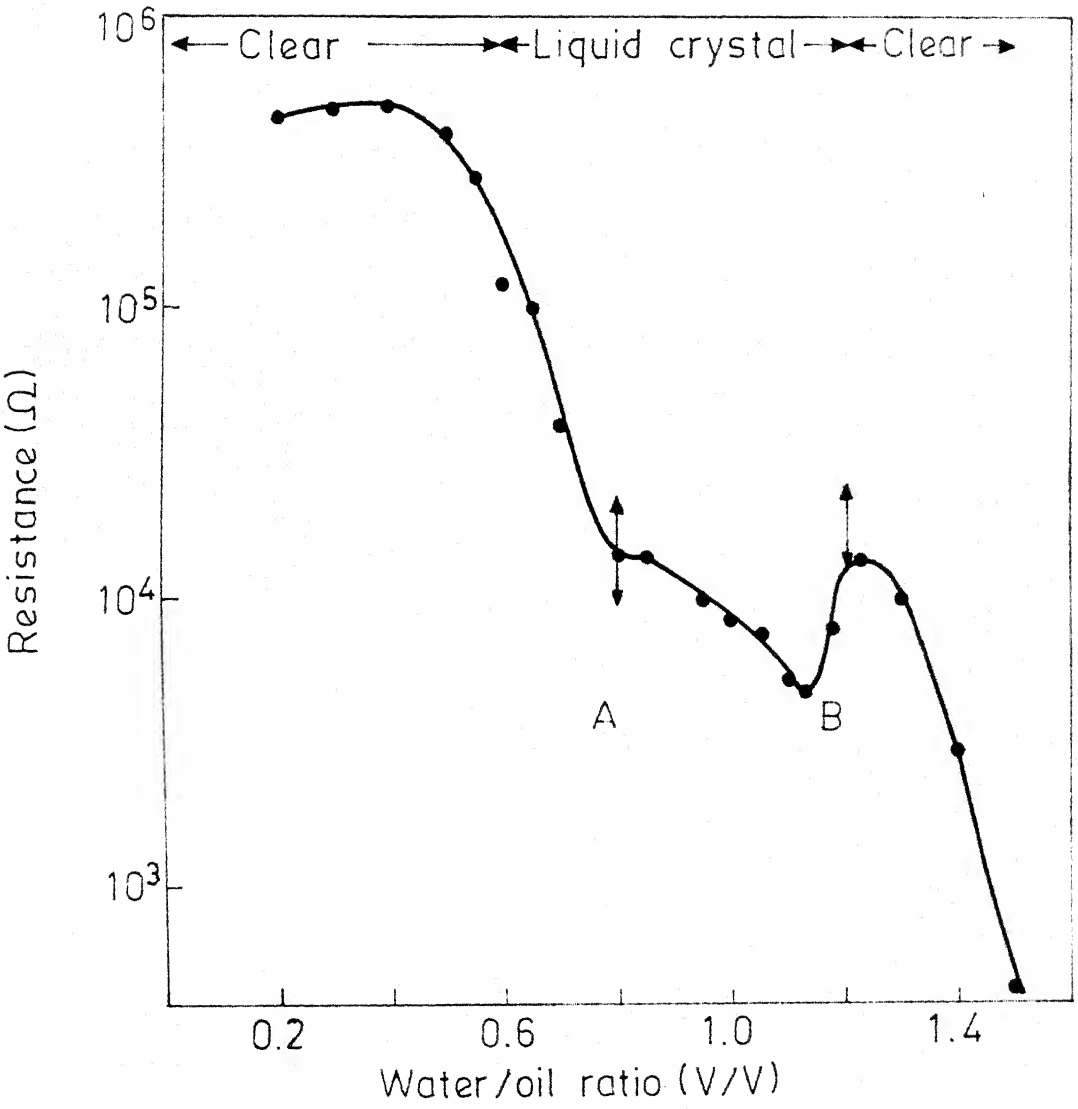


Fig. 5.2 Variation of electrical resistance of the oleate-hexanol system in presence of photochrome (Azobenzene to hexadecane molar ratio = 1:300)

measured again. It was found that irradiation has no effect on the conductivity of the model membrane in the absence of added photochromes.

Next, we prepared the same model membrane system with the photochrome azobenzene added to it at a molar ratio of azobenzene:hexadecane (1:300). The conductivity of this system was followed as a function of added water concentration. The values measured are shown as the solid curve in Fig. 5.2.

At specific water-to-oil ratios, the system containing azobenzene was irradiated for eight minutes with light of wavelength 300-400 nm, or above 400 nm wavelength. It was found that irradiation in the 300-400 nm range led to a decrease in the conductivity of the system. On the other hand, light in the 400-500 nm range led to an increase in the conductivity of the system, compared to the control values. The irradiated system, on standing, reverted back to conductivity values of the unirradiated system.

Arrows A & B at the water to oil ratio of 0.7:1 and 1.2:1 in Fig. 5.2 show the variation in conductivity of the system upon irradiation. The changes in conductance observed with irradiation at one wavelength region could be reversed by irradiation at the other wavelength region and this process could be repeated cyclically.

Azobenzene exists as an equilibrium mixture of 20% cis and 80% trans isomers roughly at room temperature. When the mixture is irradiated with light in the 300-400 nm range, the trans

azobenzene photoisomerises to cis. This produces a new photostationary state with the azobenzene almost entirely in the cis form. This photoisomerisation alters the molecular arrangement of the model membrane, as reflected by the decrease in conductivity. If the irradiation is in the 400-500 nm region, the azobenzene is converted to nearly all trans and the conductance of the system increases, signifying different changes in the molecular organisation of the model membrane. If the irradiated system is allowed to stand in the dark, azobenzene thermally reverts back (11) to its equilibrium conformer population. This changes the conductivity values back to their unirradiated values, falling on the solid line in Fig. 5.2.

In the clear water-in-oil and oil-in-water microemulsion regions of the system, conductance changes produced upon irradiation of the azobenzene containing system were much smaller in magnitude in comparison with those obtained in the liquid crystalline phase. The other photochrome used, 4,4'-azobenzenedicarboxylic acid dimethyl ester also produced results similar to azobenzene.

From the results so far presented, it is clear that the conformation of the photochrome, present at a low molecular concentration, can affect the molecular organisation of the model membrane significantly. A similar result has been reported by Verma et al., who investigated phospholipid bilayers in presence of cis and trans retinal by ESR (12). They found trans retinal to increase the order and cis retinal to decrease order in this

model membrane system.

Another observation clearly brought out the effect of the isomerisation of photochrome on the molecular organisation of the model membrane. When the system was taken at the borderline between the water-in-oil microemulsion and the liquid crystalline phase (at a water to oil ratio of 0.7:1) and irradiated to photoisomerise cis azobenzene to trans, the system changes from the liquid crystalline to the water-in-oil microemulsion, as monitored by optical anisotropy. If the photochrome was now converted to cis, the system reverted back to the liquid crystalline phase.

Since a higher degree of order in the liquid crystalline phase can be expected to lead to higher resistance, and since trans azobenzene seems to destablise the liquid crystalline phase with respect to the water-in-oil microemulsion, it seems likely that trans conformation of the photochrome promotes disorder in the model membrane and cis conformation promotes order.

Having thus established that the molecular organisation of the model membrane is sensitive to the conformation of an imbedded photochrome, the next query that naturally arises is: can these changes in the molecular organisation be further transmitted to an enzyme that is embedded in the membrane? It is well known that membrane-bound enzymes and transport systems show significant dependence in their properties on the fluidity of the membrane lipids (13). Hence, it seems possible that changes in the molecular organisation of the model membrane can be further transmitte

to an enzyme system interacting with the membrane.

The enzyme we have utilised for testing the above hypothesis was α-chymotrypsin. The monitor used to follow the effect of membrane organisation on the enzyme was the catalytic activity of the enzyme itself.

First, to test the direct effect of the photochromes on the enzyme, we assayed the enzyme in pH 8.0 phosphate buffer in the presence and absence of saturating amounts of the photochromes used. Since at ambient temperature the photochromes exist as a mixture of the cis and trans forms, the assay in presence of the photochromes should be sensitive to the interaction of the photochromes in either isomeric form with the enzyme. It was found in these assays that in presence of 4,4'-azobenzene dicarboxylic acid dimethyl ester the enzyme activity was depressed by about 20% in comparison with the pure buffer control. Since there exist many model systems in which a photochrome interacts directly with an enzyme (7), this system was not investigated any further. On the other hand, azobenzene did not affect the activity of the enzyme in aqueous buffer in any significant way. This means that either azobenzene does not bind the enzyme directly or, if it does bind, the binding is of such a nature as to not affect the activity of the enzyme in any significant way. Hence, azobenzene was used as the photochrome of choice in further studies.

α-Chymotrypsin, in either aqueous buffer or the liquid crystalline phase of the potassium oleate system, did not show

any changes in activity upon irradiation with light of either 300-400 nm or above 400 nm wavelength. This establishes that α-chymotrypsin is not intrinsically sensitive in light.

Lastly, the enzyme was assayed at a water-to-oil ratio of 1:1, corresponding to the lamellar liquid crystalline phase, in presence of azobenzene (azobenzene:hexadecane molar ratio of 1:100). The assay was carried out in presence of 3 mg α-chymotrypsin per ml of water. The assays were carried out on identical samples without any irradiation, after irradiation with 300-400 nm radiation for 10 minutes and after irradiation with above 400 nm radiation for 10 minutes.

If the activity of α-chymotrypsin in the unirradiated liquid crystalline phase in presence of azobenzene was taken as the control unit, irradiation in the 300-400 nm region (to induce trans to cis isomerisation of the photochrome), leads to a four-fold increase in the activity of the enzyme. Similarly, when irradiated in the 400 to 500 nm region to induce cis to trans isomerisation, the activity measured of the enzyme dropped to about 60% of the unirradiated control. Though there were small variations in the magnitude of the enhancement and depression of enzyme activity between different samples, the general trends were always maintained. The results of a typical enzyme assay are presented in Fig. 5.3.

Since it has already been established that the photochrome used does not interact directly with chymotrypsin, the above presented results can only be explained by the interaction of

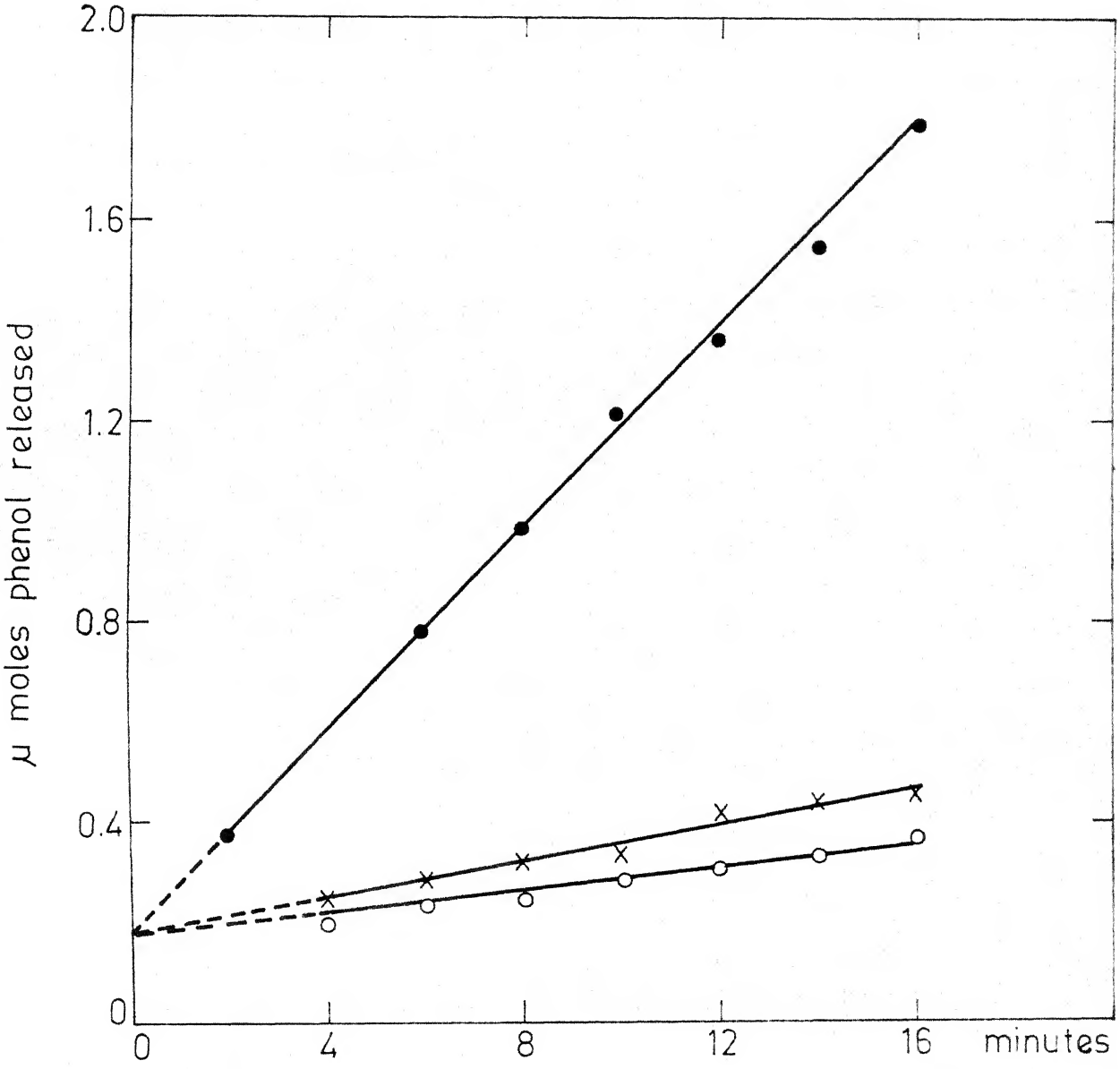


Fig. 5.3 Typical plot for assay of enzyme in model membrane system (28 ± 1°C, water to oil ratio 1:1, 35 mg enzym per ml water, azobenzene to hexadecane molar ratio = 1:1
Photochrome conformation:

- ● all cis
- o all trans
- x equilibrium mixture

the membrane system with the enzyme. The enzyme activity is modulated by the photochrome indirectly, through the membrane:

$$\text{Light} \longrightarrow \text{Photochrome} \longrightarrow \text{Membrane} \longrightarrow \text{Enzyme.}$$

This provides a very interesting model of general membrane mediated photocontrol of enzymes. The changes occurring in the physical state of the membrane may affect the enzyme conformation and hence, activity directly in the case of membrane bound enzymes (13). Another possible way in which apparent modulation of enzyme activity may be achieved is through changes in transport characteristics like the barrier to diffusion at the interfaces of the membrane with respect to the enzyme substrates or products. This can, in principle lead to the establishment of concentration gradients of substrates or products and thereby alter the kinetics of the enzyme catalysed reaction.

Membrane mediated photocontrol is very likely to occur in and is of relevance to many systems. For example, the plant physiological control protein, phytochrome, is sensitive to red radiation and exhibits photochromism:

$$\text{P red} \underset{\text{far red 720 nm}}{\overset{\text{red 660 nm}}{\rightleftharpoons}} \text{P far red}$$

The conversion of phytochrome from its red to far red form by photoisomerisation leads to a number of specific responses (14). These responses can be abolished by reisomerising phytochrome to its red form by far red irradiation. Considering the

large number of responses that quickly follow photoisomerisation of phytochrome, a membrane mediated control process would seem likely.

Similarly, the vision response is triggered by the photoisomerisation from 11 cis to all trans of the retinal prosthetic group of the vision protein, rhodopsin. Following this isomerisation, the all trans retinal is released by the protein. This step in a still unknown way, leads to set of complex responses that initiate the process of perception of light (15). One possibility, in the light of the model membrane studies discussed in this chapter, is that the retinal released by the protein interacts with the lipid matrix of the membrane. This can lead to changes in membrane order and mobility. These changes can then be transmitted through the membrane to distally located enzymes. The activity changes of these enzymes, along with changes in the permeability of the membrane may trigger the biochemical processes necessary for the perception of light. Of course, the protein may also undergo conformational changes upon release of all trans retinal. These changes, by altering protein lipid interaction, can also affect lipid order and fluidity of the retinal membrane in a way similar to the photochrome.

REFERENCES

1. B.F. Erlanger, Ann. Rev. Biochem., 45, 267 (1976).
2. H. Kaufman, S.M. Vratsanos and B.F. Erlanger, Science, 162, 1487 (1968).
3. J. Bieth, S.M. Vratsanos, N.H. Wasserman , B.G. Cooper and B.F. Erlanger, Biochemistry, 12, 3023 (1973).
4. E. Bartels, N.H. Wasserman and B.F. Erlanger, Proc. Natl. Acad. Sci. USA, 68, 1820 (1971).
5. G. Montagnoli, S. Monti, L. Nannicini and R. Felicioli, Photochem. Photobiol., 23, 29 (1976).
6. A. Ueno, H. Yoshimura, R. Saka and T. Osa, J. Amer. Chem. Soc., 101, 2779 (1979).
7. D.H. Hug in 'Photochem. Photobiol. Rev.' (Ed. K.C. Smith), Plenum Press, 1978, Vol. 3, pp. 1.
8. D.O. Shah and R.M. Hamlin, Jr., Science, 171, 483 (1971).
9. M.L.Bender, F.O. Kezdy and F.C. Wedler, J. Chem. Educ., 44, 84 (1967).
10. D.O. Shah, Ann. N.Y. Acad. Sci., 204, 125 (1973).
11. D.L. Ross and J. Blanc. in Techniques in Chemistry, Vol. III (Ed. G.H. Brown), Ch. 5 (Wiley Interscience, New York) (1971).
12. S.P. Verma, H. Schneider and I.C.P. Smith, Arch. Biochem. Biophys., 162, 48 (1974).
13. S.J. Singer in 'Structure and Function of Biological Membranes' (Ed. L.I. Rothfield), p. 145, Academic Press, New York, 1971.
14. 'Phytochrome' (Ed. K. Mitrakos and W. Shropshire Jr.) Academic Press, New York, 1972.
15. C.R. Worthington, Ann. Rev. Biophys. Bioenerg., 3, 53 (1974).

FUTURE WORK

The work contained in this thesis was envisaged and carried out with a two-fold purpose: (a) to train the candidate in and to provide exposure to various techniques of investigation which are currently found useful in investigating supramolecular assemblies, (b) to develop the basic expertise necessary in order to formulate microemulsion based model systems of a more sophisticated nature.

After the completion of the work contained in this thesis, we are currently attempting to investigate the following questions:

(1) It is well known that chlorophyll, the photosynthetic pigment, exists under certain conditions in aggregates which possess many interesting properties. Chlorophyll, in plants, occurs in photosynthetic membranes at high concentrations, presumably, in an aggregate form. J.P. Mittal has shown that chlorophyll aggregates can be stabilised in water by surfactant micelles. Can microemulsions either based on or containing chlorophyll be produced? What will be the properties of such systems in relation to chlorophyll in simple solutions?

(2) Catalysis by micelles and reverse micelles are well studied. An aspect that should be of great interest is the development of optically selective catalysts by the utilisation of optically active surfactants. Micellar catalysis by

optically active surfactants, in the studies done so far, has not shown high selectivity. We are attempting to develop reverse micelles and microemulsions from optically active surfactants. These may prove to be optically more selective as catalysts.

Other than work directed towards investigating the above two possibilities, we are also planning to continue our spectroscopic studies, especially magnetic resonance investigations, into the structure of reverse micelles and microemulsions.

LIST OF PUBLICATIONS BY THE CANDIDATE

Research:

1. Modification of a model membrane structure by embedded photochrome,
   D. Balasubramanian, S. Subramani and C. Kumar,
   Nature, 254, 252-4 (1975).

2. Studies on the Triton X-100 : alcohol:water reverse micelles in cyclohexane,
   C. Kumar and D. Balasubramanian,
   J. Colloid. Interface Sci., 69, 271-9 (1979).

3. Spectroscopic studies on Triton X-100 - alcohol microemulsions,
   C. Kumar and D. Balasubramanian,
   J. Colloid. Interface Sci. (In Press).

4. Magnetic resonance studies on the structure of a nonionic microemulsion,
   C. Kumar and D. Balasubramanian,
   J. Phys. Chem. (Manuscript submitted).

5. Proteins in microemulsions,
   C. Kumar and D. Balasubramanian,
   Nature (Manuscript submitted).

Review:

Recent Studies on the CD and ORD of Biopolymers,
D. Balasubramanian and C. Kumar,
Appl. Spectr. Reviews, 11, 223-86 (1976).